Food Research

IN THIS ISSUE

Heat Processing of Beef. Part VI. Thermal Diffusivity and "Slopes" of Heating and Cooling Curves for the High-Temperature Process

Heat Processing of Beef. Part VII. Residual Heating Effects in Beef Processed at High Retort Temperatures

Role of pH in the Canning of Mangoes: Effect of Adding Acid or Other Fruits to the Canned Product

The Formation and Decomposition of Green Pigment in Crushed Garlic Tissue

Organic Acids in Peaches

Freeze-Dried Meat. II. The Mechanism of Oxidative Deterioration of Freeze-Dried Beef

On the Nature of Cellulose from Cranberry Pulp

Effect of Moist and Dry Heat Cooking on Vitamin Retention in Meat from Beef Animals of Different Levels of Fleshing

The Hydrolysis of Beef Proteins by Various Proteolytic Enzymes

Effect in Surface Ripened Cheese of Irradiation on Spores and Toxin of *Clostridium Botulinum* Types A and B

A Critical Comparison of the Two-Sample and Triangular Binomial Designs

The Calcium and Phosphorus Contents of Some Foreign and Domestic Canned Sardines

The Fate of Ingested Polyoxyethylene (20) Sorbitan Monostearate in Rats

The Effect of Sunlight on Crude Lipids Extracted from Fresh and Frozen Vegetables

Effect of the Egg Shell Membrane on Bacteria

Notes and Letters (A Study of the Proteinases of Lipase B)

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CONTENTS

HEAT PROCESSING OF BEEF. PART VI. THERMAL DIFFUSIVITY AND "SLOPES" OF HEATING AND COOLING CURVES FOR THE HIGH-TEMPERATURE PROCESS. H. Hurwicz and R. G. Tischer 147

HEAT PROCESSING OF BEEF. PART VII. RESIDUAL HEATING EFFECTS IN BEEF PROCESSED AT HIGH RETORT TEMPERATURES. H. Hurwicz and R. G. Tischer . . 156

ROLE OF pH IN THE CANNING OF MANGOES: EFFECT OF ADDING ACID OR OTHER FRUITS TO THE CANNED PRODUCT. G. S. Siddappa and B. S. Bhatia 163

THE FORMATION AND DECOMPOSITION OF GREEN PIGMENT IN CRUSHED GARLIC TISSUE. M. A. Joslyn and Tatsuo Sano 170

ORGANIC ACIDS IN PEACHES. J. J. David, B. S. Luh, and G. L. Marsh 184

FREEZE-DRIED MEAT. II. THE MECHANISM OF OXIDATIVE DETERIORATION OF FREEZE-DRIED BEEF. A. L. Tappel 195

ON THE NATURE OF CELLULOSE FROM CRANBERRY PULP. Emmett Bennett . . . 207

EFFECT OF MOIST AND DRY HEAT COOKING ON VITAMIN RETENTION IN MEAT FROM BEEF ANIMALS OF DIFFERENT LEVELS OF FLESHING. Sylvia Cover and W. H. Smith, Jr. 209

THE HYDROLYSIS OF BEEF PROTEINS BY VARIOUS PROTEOLYTIC ENZYMES. D. S. Miyada and A. L. Tappel 217

EFFECT IN SURFACE RIPENED CHEESE OF IRRADIATION ON SPORES AND TOXIN OF *Clostridium Botulinum* TYPES A AND B. R. O. Wagenaar and G. M. Dack . . 226

A CRITICAL COMPARISON OF THE TWO-SAMPLE AND TRIANGULAR BINOMIAL DESIGNS. F. Filipello 235

THE CALCIUM AND PHOSPHORUS CONTENTS OF SOME FOREIGN AND DOMESTIC CANNED SARDINES. Antonio da Costa and Joseph A. Stern 242

THE FATE OF INGESTED POLYOXYETHYLENE (20) SORBITAN MONOSTEARATE IN RATS. Arne N. Wick and Lionel Joseph 250

THE EFFECT OF SUNLIGHT ON CRUDE LIPIDS EXTRACTED FROM FRESH AND FROZEN VEGETABLES. Frank A. Lee 254

EFFECT OF THE EGG SHELL MEMBRANE ON BACTERIA. J. L. Stokes and W. W. Osborne 264

NOTES AND LETTERS (A STUDY OF THE PROTEINASES OF LIPASE B). R. B. Koch and C. G. Ferrari 270

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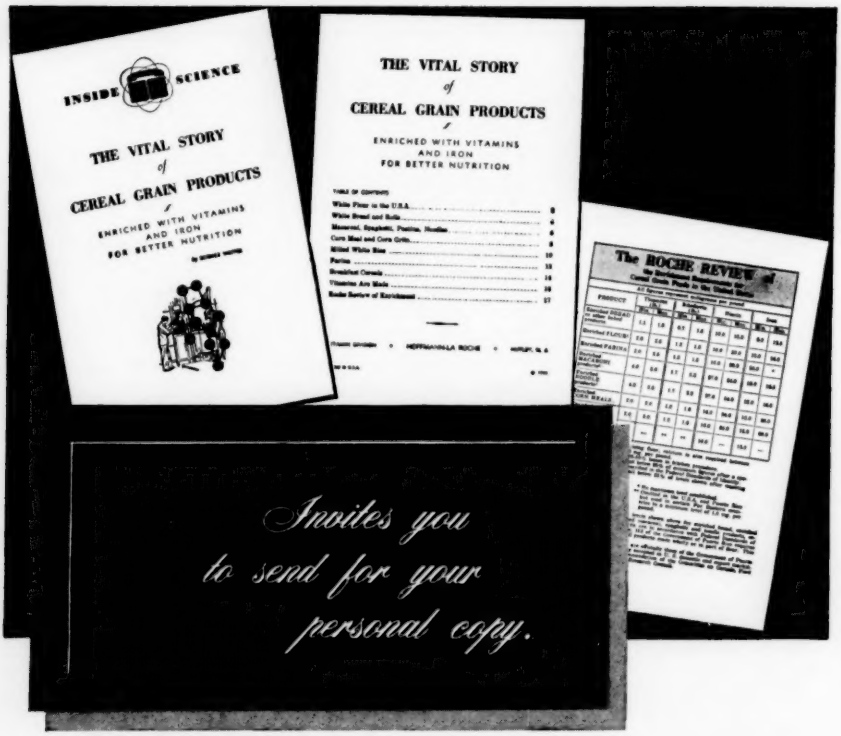

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HEAT PROCESSING OF BEEF. PART VI. THERMAL DIFFUSIVITY AND "SLOPES" OF HEATING AND COOLING CURVES FOR THE HIGH-TEMPERATURE PROCESS[a]

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(Manuscript received March 19, 1955)

The need for the determination of the physical properties of foodstuffs has been recognized in the food industry. The methods used for such determinations and for interpreting experimental results vary widely from one research study to another. It has been the considered opinion of the authors that exact mathematical and statistical methods should be used in the interpretation of results whenever applicable. This study, a part of the broad investigation reported in the "Heat Processing of Beef" series (beginning with Part III *(6)*), has for its purpose both the determination of actual physical properties of round of beef and a comparison of visual and regression methods of interpretation of the results.

The experimental design and procedure have been described in detail *(3)*. The nomenclature used in this paper is consistent with that used in earlier numbers of the series.

Thermal diffusivity (k) and its derived functions, slopes of the heating and cooling curves (f_h and f_{cl} respectively) were determined during the experiment using the visual and regression curve fitting methods. These properties of meat are of major importance in tracing the heat penetration patterns and ultimately in the evaluation of a safe canning process.

Thermal diffusivity. The slopes of the heating curves in semilogarithmic coordinates determined for the derivation of time-temperature relationships existing in the can during heat processing *(3)* gave means for the determination of thermal diffusivity of beef. Thermal diffusivity for the temperature range 225-315°F. (Fig. 1) was determined at 25 locations in the can and tabulated in Table 1 using the relationship $\hat{b} = \hat{k}(\mu_1^2 + \lambda_1^2) \log e$ derived in *(2)*, *(3)*. The variation of $\hat{k}$ was determined in terms of the coefficient of variation, $C.V.(\hat{k}) = (s_k/\hat{k}) = (s_b/\hat{b})$ in percent assuming no variation in the constant $(\mu_1^2 + \lambda_1^2)$ depending on the size of the can. The average values of thermal diffusivities for the whole space of the can are given for each processing temperature in Table 1 and the coefficients of variation in Table 2. The grand averages of Table 1 were used to plot the relationship of thermal diffusivity and retort temperature shown in Figure 1.

The average value of thermal diffusivity of beef round for the 225-315°F. retort temperature range was 0.2623 sq.cm./min.; 0.2528 for the 225-261°F. and 0.2714 for 279-315°F. temperature range. The second

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TABLE 1

Thermal diffusivities of beef ($\hat{k}$x10^5 sq. cm./min.)

Location	Retort Temperature (°F.)						
	225	243	261	279	297	315	Average
1	210.159	223.359	193.234	338.283	349.436	673.545	331.336
7	199.612	246.904	306.277	295.606	263.025	267.974	263.233
8	251.948	265.211	306.453	355.330	296.275	377.985	308.867
14	243.299	275.371	302.754	250.862	245.793	235.382	258.910
20	209.632	262.060	319.922	332.916	251.697	367.510	290.623
21	234.863	228.541	263.785	312.922	305.035	385.669	288.469
22	171.672	280.274	328.895	307.406	318.364	281.795	281.401
23	234.363	265.156	294.523	283.018	325.493	231.025	272.263
24	209.335	256.025	319.236	326.261	300.372	240.202	275.239
25	236.310	224.824	161.180	322.497	404.516	182.017	255.224
Average	220.119	252.773	279.626	312.510	306.000	324.310	282.557
2	194.096	253.541	310.050	242.547	444.672	302.911	291.303
6	192.854	284.075	294.792	263.413	232.361	243.762	251.876
9	216.927	276.863	313.433	263.951	282.194	400.825	292.366
13	259.614	276.798	261.171	238.821	375.882	309.798	287.014
15	180.062	295.291	303.997	258.111	227.977	238.274	250.619
16	247.934	304.218	286.560	262.940	218.002	185.716	250.895
17	215.110	241.908	295.126	292.557	247.878	176.576	244.859
18	250.242	280.960	292.632	250.371	216.390	197.553	248.025
19	252.013	302.707	270.895	261.142	225.112	204.625	252.749
Average	223.208	279.596	292.084	259.317	274.496	251.116	263.301
3	224.297	235.716	294.115	270.226	236.440	162.996	237.298
4	208.216	209.807	267.448	218.361	225.754	170.287	216.646
5	272.573	294.883	294.921	280.052	261.440	281.878	280.958
10	208.112	203.402	174.593	362.613	278.292	226.326	242.223
11	186.560	232.462	283.001	220.300	209.261	169.485	216.845
12	229.701	233.843	315.074	247.766	270.079	215.934	252.066
Average	221.577	235.019	271.525	256.553	246.878	204.484	241.006
Grand Average	221.634	255.796	281.078	279.460	275.791	259.970	262.288

value may be compared to the thermal diffusivity of beef clods *(2)* which was 0.113 sq. cm./min.

The tabulated data indicate a relatively small variation in the determination of diffusivity for various locations with few exceptions. This was true for all retort temperatures. The computed variation pertains to the average of 9 determinations. The variation of the mean diffusivity of 25 locations would be correspondingly lower.

The observed effect of the temperature on the average values of thermal diffusivity indicated that diffusivity is not a constant but may be a function of temperature. Thermal diffusivity was observed (Table 1 and Figure 1) to increase with the retort temperature in the range from 225-261°F. and then decrease. The same trend was observed previously *(4)* in the case of lag coefficients and intercept coefficients, and in determination of other processing variables. All these phenomena might well be affected by the changes in diffusivity of beef and indicate that the retort temperature range 261-279°F. may be located in a critical region for processing of beef. The coefficients of variations decreased in the range from 225-261°F.

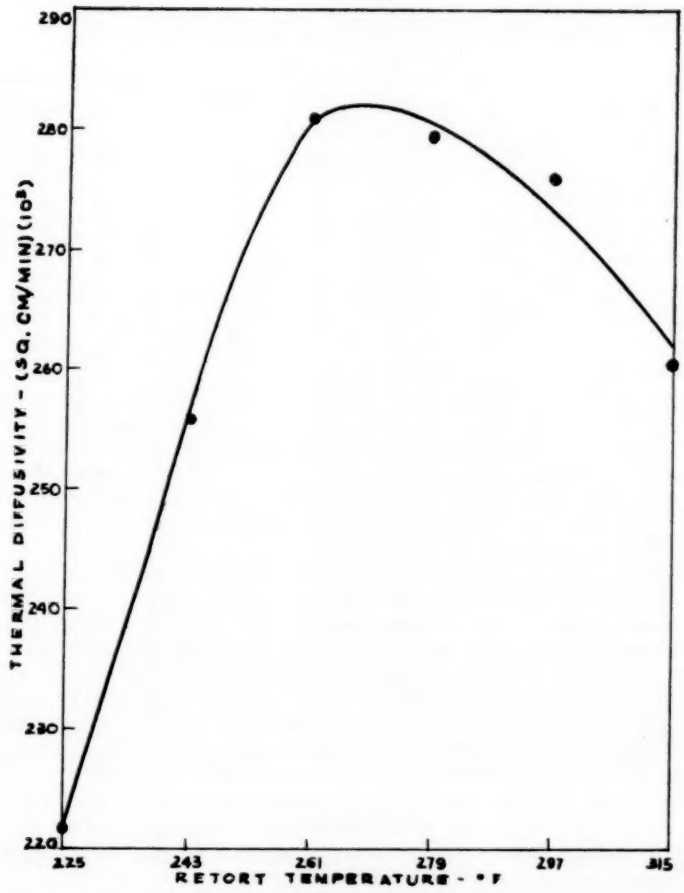

Figure 1
Relationship of thermal diffusivity of beef and retort temperature

Inspection of the values of the variance for individual determinations of diffusivity at all locations in the can and computation of confidence limits (at 1% probability level) for these determinations indicated that there were no significant differences in the diffusivity due to the location in the container. The 1% probability level for testing possible differences has been chosen because according to the laws of physics thermal diffusivity is a constant depending on the material; however, a possibility existed that chemical and moisture changes occurring during processing might have affected the thermal properties of beef to different extents depending on the distance of the region in the can from the source of heat. A high probability level was thus necessary to assure correctness of inferences drawn on the basis of statistical analysis.

TABLE 2

Average coefficients of variation of the thermal diffusivity of beef ($C.V. = s_{\hat{k}}/\hat{k}$)

Location	Retort Temperature (°F.)						
	225	243	261	279	297	315	Average
1	11.90	6.65	11.10	2.42	4.22	12.69	8.16
7	6.26	5.41	3.26	3.21	4.73	7.78	5.11
8	8.42	4.06	3.19	4.91	6.31	6.15	5.51
14	10.13	3.43	5.14	3.88	4.68	5.30	5.43
20	10.25	2.21	3.83	3.60	6.50	12.36	6.46
21	3.68	7.97	5.21	10.26	4.04	9.61	6.80
22	7.90	6.88	4.45	2.41	4.87	6.92	5.57
23	7.08	2.57	3.32	4.21	5.84	7.98	5.17
24	10.71	3.32	3.62	4.47	8.43	5.36	5.99
25	7.32	6.79	12.96	7.29	7.25	23.54	10.86
Average	8.37	4.93	5.61	4.67	5.69	9.77	6.51
2	5.19	4.92	4.19	2.19	8.84	7.84	5.53
6	8.04	2.94	2.52	3.15	7.10	5.51	4.88
9	9.11	3.45	6.46	3.44	3.22	13.48	6.53
13	7.32	2.68	3.17	16.00	10.17	20.51	9.98
15	7.09	4.12	3.17	2.18	3.57	5.60	4.29
16	7.31	5.68	5.69	3.29	2.62	6.70	5.22
17	9.98	2.71	5.90	4.32	5.76	5.30	5.66
18	8.53	4.54	5.65	4.59	3.45	8.46	5.87
19	6.70	7.20	4.72	2.33	3.06	5.93	4.99
Average	7.70	4.25	4.60	4.61	5.31	8.81	5.88
3	4.03	3.05	3.14	3.82	4.52	13.99	5.43
4	7.07	4.88	8.43	6.51	18.36	9.86	9.19
5	4.81	3.14	12.96	3.56	5.97	12.73	7.20
10	12.89	8.60	8.45	5.58	5.08	6.57	7.86
11	5.88	3.09	5.07	4.35	5.98	5.74	5.02
12	5.37	3.86	6.57	14.49	5.93	7.05	7.21
Average	6.68	4.44	7.44	6.39	7.64	9.32	6.99
Grand Average	7.58	4.54	5.88	5.22	6.21	9.30	6.46

TABLE 3

Mean values and analysis of variance of f_h for six retort temperatures

Mean Values

RT (°F.)	225	243	263	279	297	315	Average
f_h (min.)	39.30	37.32	38.19	36.31	39.94	44.04	39.184

Analysis of Variance

Source	df	S.S.	M.S.	"F"
Region	2	3634.1982	1817.10	69.68**
Temperature	5	774.4810	154.896	5.94**
R x T	10	979.4331	97.943	3.76**
Exp. Error	108	2816.3956	26.078	.....
Total	125	8204.5079	.....	.....

** Significant at 1% probability level.

TABLE 4

Mean values and analysis of variance of $\hat{f}_h$ for six retort temperatures

Mean Values

RT (°F.)	225	243	263	279	297	315	Average
$\hat{f}_h$ (min.)	41.98	36.32	33.89	33.51	33.76	40.35	36.633

Analysis of Variance

Source	df	S.S.	M.S.	"F"
Region	2	262.0160	131.0080	3.28*
Temperature	5	1429.4543	285.8909	7.15**
R x T	10	509.1297	50.9130	1.27
Exp. Error	108	4315.9949	39.9629	.....
Total	125	6516.5949	.....	.....

* Significant at 5% probability level.
** Significant at 1% probability level.

This interpretation of effects of retort temperature and of the location in the can on the diffusivity was borne out in analysis of variance of "slopes" of heating curves ($\hat{f}_h$) (Table 4) which are derived functions of diffusivity.

Consideration should be given to the possible sources of variation, other than variation in temperature measurements and lack of homogeneity of meat, in the determination of thermal diffusivity. The can factor $(\mu_1^2+\lambda_1^2)$ has been assumed to be constant in computation of coefficients of variation of $\hat{k}$ from the data on slopes ($\hat{b}_h$) of the heating curves. The variation of the latter, however, may be composed of variation in the diffusivity and in the can factor. The variation of the can factor would be negligible if the meat sealed in the container occupied its whole space at any time of processing. This condition, however, is fulfilled only at the time of closing of the can; thereafter, the piece of meat shrinks and the effective size of the can (the size of the piece of meat) is changed. The process of shrinking affects the value of the can factor which is a function of the size of the container. The assumption of constancy of the can factor might have introduced additional variation in the determination of $\hat{k}$ as computed from the values of $\hat{b}_h$.

"Slopes" of heating curves. The "slope" of a heating (or cooling) curve f_h (or f_{cl}) is defined in food technology practice as the number of minutes necessary for the heating (or cooling) curve to traverse one logarithmic cycle (Brigg's logarithms). The "slope" thus defined is actually the reciprocal of the absolute value of the slope $b=k(\mu_1^2+\lambda_1^2)\log e$ of the heating curve and as such is a function of the thermal diffusivity and of the can size.

The f_h values were determined during this experiment by two methods. One method consisted of visual fitting of a straight line to the heating data plotted on semi-log graph paper and counting the time necessary for this line to traverse one log cycle. The value thus determined was recorded as f_h. The value of f_h was obtained by taking the reciprocal of the slope of the heating curve determined by the last-squares regression method. The values of $\hat{f}_h$ and f_h were recorded for all of the locations and retort temperatures investigated. The trends displayed by the f_h and f_h values were similar to those of thermal diffusivity as had been expected,

TABLE 5

Mean values and analysis of variance of f_{cl} for six retort temperatures

Mean Values

RT (°F.)	225	243	261	279	297	315	Average
f_{cl} (min.)	38.659	38.389	37.572	43.515	40.203	40.264	39.767

Analysis of Variance

Source	df	S.S.	M.S.	"F"
Region	2	2198.8482	1099.42	32.83**
Temperature	5	471.0662	94.21	2.81*
R x T	10	351.0610	33.106	1.05
Exp. Error	108	3616.7120	33.488	.....
Total	125	6637.6874	.....	.....

* Significant at 5% probability level.
** Significant at 1% probability level.

and displayed a minimum in the middle of the 225-315°F. temperature range. Analyses of variance were made on 21 locations in the can (7 locations were chosen at random from each of the 3 regions in the can: outside, intermediate, and central) and for all 6 temperatures for $\hat{f}_h$ (Table 4) and f_h (Table 3). These analyses indicated that in both cases the effect of retort temperatures on mean values was significant at the 1% probability level. The effect of the location in the can on the f_h value was also significant at the 1% level, but the same effect was not significant for the $\hat{f}_h$ values (1% level was chosen for the reasons explained previously). The variance of $\hat{f}_h$ was found to be greater than for f_h and the homogeneity of the variance could be doubted as the "F"-test *(5)* was significant between the 10% and 2% probability levels. The average values of f_h and $\hat{f}_h$ are given in Tables 3 and 4.

"Slopes" of cooling curves. The data on values of the "slopes" (f_{cl}) of cooling curves were obtained and treated in the same manner as the heating data. The inspection of mean values of $\hat{f}_{cl}$ indicated its constancy over the retort temperature range used and in the whole space of the can. The values of f_{cl} on the contrary showed an increase in values from the outside of the can toward the center, and a maximum for the 261-279°F. retort temperatures. The analyses of variance made on these characteristics confirmed the trends observed by inspection. The "F"-values for $\hat{f}_{cl}$ were not significant for either temperature or location effect on the mean value of $\hat{f}_{cl}$ (Table 6). The contrary was true for f_{cl} analysis (Table 5). "F"-tests indicated a very significant effect of location at the 1% probability level and significant (at 5% level) effect on mean values of the temperature. The variance of $\hat{f}_{cl}$ was found to be very large (542.458) and considerably larger than that of f_{cl} (33.488). These variances were not homogeneous. The average values of f_{cl} and $\hat{f}_{cl}$ are given in Tables 5 and 6.

Comparison of the "slopes" of heating and cooling curves. The values of the "slopes" of heating and cooling curves were compared to determine whether they could be considered to be equal. The use of f_h value for the "slope" of the cooling curve was recommended in Ball's "formula

TABLE 6
Mean values and analysis of variance of $\hat{f}_{el}$ for six retort temperatures

Mean Values

RT (°F.)	225	243	263	279	297	315	Average
$\hat{f}_{el}$ (min.)	40.000	42.736	51.486	42.873	46.532	54.150	46.296

Analysis of Variance

Source	df	S.S.	M.S.	"F"
Region	2	2219.440	1109.720	2.0457
Temperature	5	3206.792	641.358	1.1823
R x T	10	5376.423	537.642	0.9911
Exp. Error	108	58585.484	542.458	.....
Total	125	69388.139	.....	.....

method" of process determination *(1)*, although Ball himself expressed doubts whether such a procedure was justified.

The comparison of mean $\hat{f}_h$ vs. $\hat{f}_{el}$ and of f_h vs. f_{el} values indicated by means of "t"-tests that in both cases the means differ significantly (at 1% probability level) thus giving evidence that $\hat{f}_h$ and $\hat{f}_{el}$ were not equal and cannot be used interchangeably. In addition the variances of $\hat{f}_{el}$ and $\hat{f}_h$ were not homogeneous, the first one being considerably larger. This finding is in agreement with theoretical expectations discussed in the section on cooling phase of the canning process in Part IV *(3)* of this series. The corresponding values estimated visually were found substantially the same.

Comparison of the visual and regression estimates of "slopes." The superiority of regression methods of curve fitting as compared with the visual methods has been emphasized repeatedly in the discussion of results obtained during the investigations reported in this series *(2, 3, 4)*. The comparison of results of "slope" determinations and possible inferences drawn from them, dependent on the method of curve fitting, may serve as an example.

Since in this case both the visual and regression method estimate the same straight line relationship, the least squares method gives the better fit by its definition. The results obtained by the visual method can only approximate the results of the regression method. The inference drawn from the regression may be then considered as a true picture of the condition, whereas some doubt may be raised with respect to the inference drawn from the results of visual approximation. Therefore, if results obtained by both methods are available, the regression data should be considered as the standard of comparison and basis for drawing conclusions. The same is true with respect to the estimation of experimental variation. It may be noted at this time that there is no estimate of variation of the slope by visual methods when only one line is fitted, whereas an estimate is available when the regression method is used.

From the comparison of $\hat{f}_h$ vs. f_h discussed above it was observed that their means were significantly different and that the variance was most likely not homogeneous. On the basis of the analysis of variance one concluded that $\hat{f}_h$ did vary with temperature but not with the location

in the can; on the other hand the inference drawn from analysis of variance for f_h would indicate that the location in the container had a paramount effect on the "slope" of the heating curve. If the visual estimate were the only one available, incorrect conclusions could have been drawn. A similar situation was found to exist for the estimates of "slopes" of cooling curves. Analysis of variance indicated highly significant effects of location in the can and significant effects of retort temperature on the mean values of f_{cl}. The picture presented in the analysis of variance for $\hat{f}_{cl}$ did not show any such effects. Since the means of $\hat{f}_{cl}$ and f_{cl} were significantly different, again incorrect conclusions would be drawn on the basis of visual estimates.

The comparison of the values of "slopes" of heating and cooling curves gave the same results for both methods. Nevertheless, the visual estimate indicated a homogeneous variance for f_h and f_{cl}, whereas the regression estimates showed a distinctly different variance for $\hat{f}_h$ and $\hat{f}_{cl}$ and bore out the fact that these two properties are not only different but also that their variations might come from different sources. That this was the case can be deduced from the discussion of the cooling phase *(3)* where the value of $e^{(t-t_1)/f_{cl}}$ was found to depend on the value of the g/m ratio which is also subject to variation.

The above discussion showed that visual estimation of the "slopes" of the heating and cooling curves may lead to erroneous results and conclusions, and that it is imperative to estimate these parameters by means of least squares regression for specification of a thermal process to produce a palatable and safe canned beef product.

CONCLUSIONS

On the basis of experimental evidence and its statistical interpretation it may be concluded that:

Thermal diffusivity does not depend on the location in the container; it may be, however, a function of processing temperature and reaches a maximum for 261-279°F. ($k_{aver.} = 0.2623$ sq. cm./min.).

The "slopes" of the heating and cooling curves, f_h and f_{cl} are significantly different and cannot be used interchangeably. The visual estimates of these "slopes" are significantly different from their regression estimates and should not be used in process determination.

In addition, the experimental findings in this study confirm trends observed in *(4)* and indicate that processing temperatures between 261-279°F. display the characteristics of a critical range where most of the physical characteristics assume their extremes. This temperature range may prove to be optimal for canning of safe and palatable beef.

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HEAT PROCESSING OF BEEF. PART VII. RESIDUAL HEATING EFFECTS IN BEEF PROCESSED AT HIGH RETORT TEMPERATURES[a]

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The lag in cooling observed immediately after the end of the heating phase has been long recognized in the studies of heat penetration into cylindrical containers undergoing a thermal process. Most research workers investigated the center of the can, and several attempts were made *(1, 2)* to find a mathematical formula expressing the time-temperature relationship in the early stages of cooling. The formulae derived assumed this relationship to be (in terms of degrees above the cooling water temperature) a monotonic nonincreasing function from the beginning of cooling, or time when cooling water was started and steam cut off. This assumption might have been justified for all practical purposes for low-temperature, long-time heating processes; however, it has been observed by Hicks *(3)* and by the present authors in the past in *(4)* and *(5)* that for relatively short processes and especially for high temperatures, this assumption is not justified by the experimental facts. Furthermore, the theoretical relationship (eq. 1 *(5)*) in its series expansion indicates that for small values of $(t-t_1)$ a certain amount of residual heating is to be expected in the central region of the can. In the expression $(t-t_1)$ t_1 is the designated process time; t is any time after the beginning of process.

The experimental procedure and design of this investigation have been described in detail in previous papers *(5, 8)*. The nomenclature used in this work is consistent throughout with the same references. New symbols introduced for the first time are explained in Table 1.

Intermediate cooling period. The intermediate cooling was defined by the authors *(5)* as the period occurring immediately after the steam is shut off and cooling started, and before the time-temperature relationship is approximated by a linear function in semi-log coordinates. This period corresponds to the introductory phase of heating but assumes a more important role in process lethality calculation because of high temperatures involved. Experimental evidence from the investigation used for derivation of a time-temperature relationship during the intermediate cooling showed that more than one term in the series expansion of Equation 1 *(5)* should be retained to approximate the actual thermal history in the can, and that some rise in the temperature for small $(t-t_1)$ occurred. Therefore, the function to be derived should not be monotonically decreasing. The occurrence of residual heating affects significantly the

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TABLE 1

Effects (min.) of residual heating at the point of greatest heating lag for six retort temperatures (RT)

RT = 225°F. t_2(a) = 103 min.				RT = 243°F. t_2 = 88 min.			
(b) t_1	(c) Δt	(d) $\Delta t'$	(d) $\Delta t/\Delta t'$	t_1	Δt	$\Delta t'$	$\Delta t/\Delta t'$
10	12.00	93	0.129	8	14.00	80	0.175
20	6.00	83	0.072	17	6.56	71	0.092
30	3.72	73	0.051	25	4.44	63	0.070
40	3.00	63	0.048	34	2.11	54	0.039
50	1.66	53	0.031	42	1.66	46	0.036
60	2.44	43	0.057	50	1.11	38	0.029
70	0.33	33	0.010	59	2.00	29	0.067
80	0.33	23	0.014	67	1.33	21	0.063
90	0.00	13	0.000	76	0.00	12	0.000
100	0.00	3	0.000	84	1.22	4	0.030
110	1.00	....	.....	93	0.00	....	.....
120	0.00	....	.....	101	0.66	....	.....
Average			0.041	Average			0.060

RT = 261°F. t_2 = 74 min.				RT = 279°F. t_2 = 59 min.			
t_1	Δt	$\Delta t'$	$\Delta t/\Delta t'$	t_1	Δt	$\Delta t'$	$\Delta t/\Delta t'$
7	13.28	67	0.198	5	12.66	54	0.234
14	7.78	60	0.130	10	10.00	49	0.204
21	8.06	53	0.152	16	8.00	43	0.186
27	4.44	47	0.094	21	5.33	38	0.140
34	3.89	40	0.097	26	5.17	33	0.157
41	4.00	33	0.121	31	2.78	28	0.092
48	2.00	26	0.077	36	1.89	23	0.080
54	4.00	20	0.200	42	3.00	17	0.176
61	0.78	13	0.060	47	0.00	12	0.000
68	1.11	6	0.185	52	1.55	7	0.221
75	0.00	....	.....	57	0.33	2	0.165
87	0.33	....	.....	62	0.33	....	.....
Average			0.131	Average			0.150

RT = 297°F. t_2 = 44 min.				RT = 315°F. t_2 = 29 min.			
t_1	Δt	$\Delta t'$	$\Delta t/\Delta t'$	t_1	Δt	$\Delta t'$	$\Delta t/\Delta t'$
3	9.89	41	0.241	2	13.77	27	0.510
7	8.22	37	0.222	4	10.50	25	0.420
11	8.44	33	0.256	6	9.89	23	0.430
14	7.66	30	0.255	8	9.66	21	0.460
18	5.66	26	0.218	10	10.50	19	0.553
21	4.66	23	0.203	12	9.33	17	0.549
25	5.00	19	0.263	14	5.33	15	0.355
29	4.11	15	0.274	16	8.17	13	0.628
32	3.22	12	0.268	18	4.00	11	0.333
36	3.33	8	0.416	20	4.33	9	0.481
40	0.00	4	0.000	22	2.17	7	0.310
43	0.50	1	0.500	24	3.76	5	0.752
Average			0.260	Average			0.482

(a) t_2 is the time when $\Delta t = 0$.
(b) t_1 is the designated processing time.
(c) $\Delta t = t'_1 - t_1$ = (effective processing time) − (designated processing time).
(d) $\Delta t' = t_2 - t_1$.

sterilizing effect (lethality) of the process, and thus is of great importance in canning process evaluation. These effects have been evaluated and will be discussed at length.

In the attempt at the determination of functional relationship of time, temperature, and space during the intermediate cooling, average thermal histories at 12 locations chosen at random from the central, intermediate, and outside regions of the can were compared with the theoretically expected temperatures at these locations. Fifteen terms ($i = \ldots, 5$ and $m = 1, \ldots, 5$) of Equation 1 given in *(5)* were calculated for retort temperatures 243, 279, and 315°F. and for 4 values of $(t - t_1)$ ranging from 2 to 14 minutes. For each of the locations temperatures were calculated for cooling curves starting at 3 values of $(RT - CT)_{t_1}$ (difference between retort temperature and can temperature at a given location at time t_1—equivalent to Ball's g) to estimate the influence of $(RT - CT)_{t_1}$ on the necessary number of terms to be retained in the expansion without affecting significantly the accuracy of the prediction. Since the design of this experiment *(5)* did not have as a main objective the derivation of prediction equations for the intermediate cooling phase, only approximate comparisons could be made and only tentative conclusions may be drawn. These comparisons were made only by inspection of the corresponding experimental and theoretically computed data.

From inspection of the experimental data certain trends were observed. For the locations tested it appeared from the computed data that for values of $(t - t_1) > 10$ minutes the inclusion of more than one term of the series did not change the values of temperatures by more than 1°F. Furthermore, for the values of $(t - t_1)$ of 2-3 minutes the series was observed to converge to the limit after 12 terms were computed; for $(t - t_1)$ of 5-7 minutes the limit was reached with approximately 7-8 terms computed, and only 4 terms were necessary for $(t - t_1) = 10$ minutes. The number of terms required for attaining the limit was not influenced by the retort temperature but fewer terms had to be used on the average when the value of $(RT - CT)_{t_1}$ decreased. Since $(RT - CT)_{t_1}$ was smaller for outside locations, fewer terms are required in this region than in the interior part of the container. Direct comparison of computed and experimentally observed temperatures indicated overestimate of the temperature by the theoretical equation for $(t - t_1) < 3$ minutes and underestimate thereafter. Thus, the observed rise in temperature after cooling water was started was not reflected properly by the theoretical estimates.

The results of these evaluations of the trends during the intermediate cooling phase do not warrant a recommendation of the prediction equation for the time-temperature relationship in the can. It may be concluded, however, that the theoretical equation does not approximate well the thermal history in the can as assumed by Hicks *(3)* since the series converges to a temperature value not in agreement with the experimental data. The temperatures observed indicate definitely that the temperature-time relationship is not a monotonic function as assumed by Ball *(1)*. This is especially true for large values of $(RT - CT)_{t_1}$ and thus for the high-temperature, short-time process. It would seem that a separate investigation of the above functional relationship may be warranted by the importance of the intermediate cooling phase for canning process determination.

Residual heating effect. In the discussion of time-temperature relationships during the intermediate cooling phase, attention was drawn to the effects of residual heating resulting in increase in temperature at locations in the can at the beginning of the cooling phase of the process. Since no prediction equation was derived to represent the functional relationship for that period, some of the effects of residual heating are discussed and illustrated graphically in the following section.

The end of the heating phase determines the processing time equal to t_1; the residual heating extends the effective processing time by an interval Δt to t'_1. Processing time requirement calculated from the thermal history at the center of the can (or point of greatest heating lag *(6)*) by methods which neglect the effect of residual heating at this location *(1)* specifies actually t'_1 and not the t_1. Thus a calculated process requirement is an overestimate of the retort holding time. In view of the above the determination of $\Delta t = t'_1 - t_1$ becomes of interest as it may lead to shorter safe processing times, and thus improve the palatability of the canned product.

In an attempt to determine the effective heating time (t'_1), Δt was measured at the location of greatest heating lag in the container for several processing times (t_1) at each of the 6 processing temperatures. The results were tabulated (Table 1) and the average of 3 determinations of Δt for each processing time (t_1) was plotted against t_1. The plot of Δt for each temperature resulted in an approximately straight line relationship. The intercept of the line yielded values of time (t_2) necessary to achieve a state when $\Delta t = 0$ or $t'_1 = t_1$. In other words a processing time was determined when no residual heating took place after the steam was shut off. The values of t_2 thus obtained were plotted (Figure 1) against temperature (RT) giving an excellent fit as a straight line of the form, $t_2 = a - b(RT)$. Since it was noticed that Δt decreases with the increase in t_1, a new variable was introduced, $\Delta t' = t_2 - t_1$ (the time remaining to the moment when $\Delta t = 0$) which also decreased with the increase in t_1. The values of t_2 used in computation of $\Delta t'$ were taken from Figure 1. Next the ratio of $\Delta t / \Delta t'$ was computed for each processing time and found substantially constant for each of the 6 temperatures used in this investigation. The plot of $\Delta t / \Delta t'$ against RT (Fig. 1) was found to be a curvilinear function of second degree increasing with the increase in retort temperature.

The meaning of the increase of $\Delta t / \Delta t'$ with processing temperature may reflect two occurrences: first and more significant, the effect of residual heating becomes larger as the retort temperature becomes higher for the same value of $\Delta t'$; second, for the same residual heating effect (in terms of time) less time is necessary to reach the point when $\Delta t = 0$. From Table 2 one can readily see that t_2, as determined previously for the increasing temperatures, corresponds to rapidly increasing sterilizing values. This table also shows that for processes equivalent in terms of lethality, $\Delta t' = t_2 - t_1$, when computed for $F_o = 2$, increases from 1 minute at 225°F. to about 36 minutes at 261°F. and then decreases to 12 minutes at 315°F. This trend is in agreement with trends observed by Hurwicz and Tischer in *(6)* and *(7)* for other thermal properties of beef. The respective values for Δt are 0.04, 4.68, and 5.76 minutes, which indicates the larger and larger effect of residual heating, for processing times com-

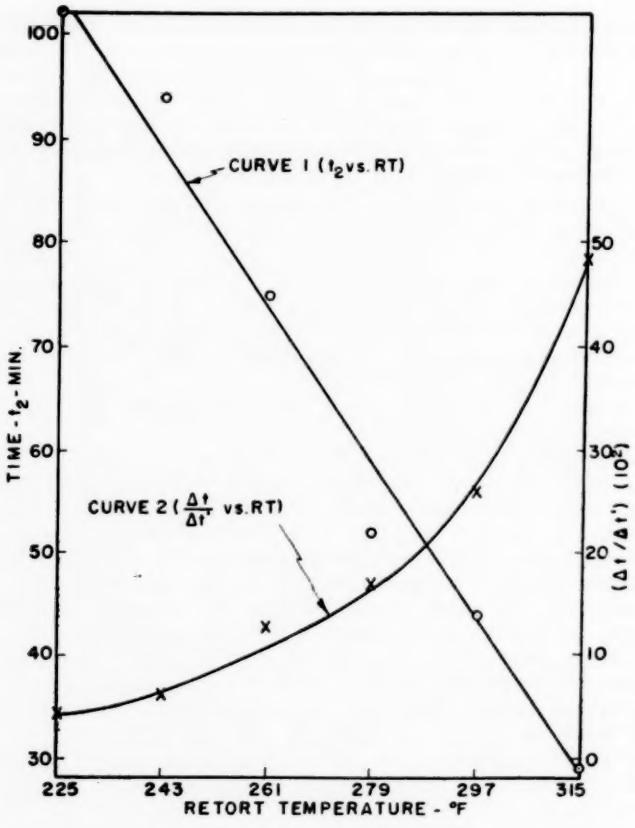

Figure I. Residual Heating Effects.
(For use in determination of the effective heating time — t'_1)

TABLE 2
Mean values of processing times t_1 (min.) for five F_o values[1]

RT (°F.)	t_2 (min.)	F_o				
		1	2	3	4	5
225	103	74.0	102.0	119.5	.....	.....
243	88	49.0	55.0	58.0	61.5	63.0
261	74	34.0	37.5	39.5	41.0	42.0
279	59	23.0	26.0	27.5	22.5	29.5
297	44	21.0	24.0	25.0	26.0	26.5
315	29	16.5	17.8	18.0	18.5	18.7

[1] Based on *(8)* Table 9.

monly used in canning, as the temperature increases. This effect is augmented by the fact that at higher temperatures the F_o values increase with time faster than at low temperatures.

The usefulness of the computed ratio $\Delta t/\Delta t'$ lies not only in facilitation of interpretation of effects of residual heating, but also gives means, with the help of Figure 1 to determine the effective heating time (t'_1) for a given process (t_1) or vice versa. The ratio $(\Delta t/\Delta t') = (t'_1 - t_1)/(t_2 - t_1)$ may be found for retort temperatures in the range 225-315°F. from Curve 2, and t_2 from Curve 1 (Fig. 1). Thus the effect of residual heating may be approximately assessed and corrections to the estimated process time by the "formula method" may be made.

The fitting of the curves by statistical methods would give means of evaluation of the precision of these results. It would seem unnecessary, however, to evaluate the precision of a procedure to be used in connection with the "formula method," since that method does not afford an estimate of precision itself, and because of large variations in F_o determinations.

SUMMARY AND CONCLUSIONS

In the course of the investigation of the intermediate cooling period and residual heating effects a method has been developed to determine effective retort holding times. This method may result in shorter but safe processing times and may increase the palatability of canned beef and other products.

On the basis of the experimental results the following conclusions may be drawn:

Neither the theoretical solution of the heat conduction equation nor a monotonic function approximate the experimental results for the intermediate cooling phase.

The residual heating at the beginning of the cooling phase increases the effective processing time. This increase is most pronounced for short time, high temperature processes. The contribution of residual heating to the sterilizing effect displays the same trend and possibly reaches maximum at 297°F. retort temperature.

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ROLE OF pH IN THE CANNING OF MANGOES: EFFECT OF ADDING ACID OR OTHER FRUITS TO THE CANNED PRODUCT

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In canning fruits, the pH of the fruit is the critical factor that determines the efficacy of the heat processing. Reliance is placed more on the low pH of the fruit than on complete destruction of all bacterial spores. Although over the pH range of 4.0-4.5, growth of acid tolerant organisms such as *Clostridium butyricum* may occur, the critical pH on which most processes are based is generally taken as 4.5. Many of the fruits have a pH range well below this limit. Among the tropical fruits, however, there are some like jack fruit, certain varieties of mangoes like *Badami* and *Mulgoa*, banana, sapota, papaya, litchi, etc., which often have a pH much above this critical limit.

Ordinary processing in boiling water is not always a safe method for canning such fruits. A well-known method for overcoming this shortcoming in canning such fruits is adding a fruit acid to the syrup to lower the pH. This is of special importance since cases of botulism have been reported in the U.S.A. in which canned tomatoes, pears, mangoes, and apricots were suspected as being the cause and in some cases were proved to be so *(1)*.

Although the importance of pH in connection with the processing and storage of canned foods is generally understood and appreciated, at present little control of pH is exercised in fruit and vegetable canning. pH not only influences the degree of survival of spoilage microorganisms during processing but also affects the germination and growth of any bacterial spores which might have withstood the process given. Recently, Adam *(1)* studied the factors affecting pH in fruit and vegetable canning and the changes that take place during processing.

Among the commercially important varieties of mangoes in South India, *Badami*, *Raspuri*, *Neelum*, *Bangalora* and *Mulgoa* are the foremost. Some of these have a pH slightly more than 4.5. It was therefore thought desirable to study their heat processing with special reference to their pH. The effect of adding acid to the syrup at different levels and also utilizing more acid fruits to lower the pH were studied. The possibility of utilizing a more acidic mango like the *Bangalora* to can a less acid fruit like jack fruit or banana was also investigated.

EXPERIMENTAL

Material and methods. The mangoes used in these studies were fully mature, firm, and at the canning-ripe stage. The pulp was prepared by using a Waring blender. The pH of the pulp or syrup was measured by means of a Beckman Glass Electrode pH meter. The acidity of the syrup as well as of the drained fruit was determined by titration with $\frac{N}{10}$ NaOH solution. The different sets of samples, canned by the usual procedure, were stored at room temperature of 24-30°C. and then examined at the end

TABLE 1

Effect of added acid on the pH of mango pulp and sugar syrup mixtures.

Raspuri Mango						Badami Mango					
Pulp		Pulp + 50° Brix syrup (2:1)		Pulp + 35° Brix syrup (2:1)		Pulp		Pulp A + 50° Brix syrup (2:1)		Pulp B + 50° Brix syrup (2:1)	
Added acid	pH	Added acid	pH	Added acid	pH	Added acid	pH	Added acid	pH	Added acid	pH
%		%		%		%		%		%	
0.00	3.60	0.00	3.60	0.00	3.65	0.00	3.80	0.00	3.80	0.00	4.90
0.24	3.40	0.16	3.40	0.16	3.30	0.24	3.65	0.16	3.65	0.39	4.10
0.56	3.30	0.39	3.25	0.31	3.25	0.46	3.50	0.31	3.50	0.71	3.85
1.00	3.20	0.71	3.00	0.46	3.20	0.65	3.40	0.46	3.40	1.00	3.60
1.36	3.10	1.00	2.95	0.59	3.10	0.83	3.25	0.59	3.30	1.25	3.45
1.67	2.95	1.25	2.85	0.71	3.00	1.00	3.20	0.71	3.25	1.47	3.35
(0.5% citric acid solution only)	2.15			0.83	2.95	1.36	3.05	1.00	3.05	1.67	3.15
				0.95	2.95	1.67	3.00	1.25	2.90		
				1.05	2.95						
				1.15	2.90						
				1.25	2.90						
				1.67	2.75						
				2.00	2.70						

of different periods of storage. Cut-out data regarding drained weight of fruit, Brix of syrup, etc., were also collected.

RESULTS AND DISCUSSION

Addition of citric acid. To study the buffer action of mango pulp, increasing quantities of citric acid were added to *Badami* and *Raspuri* mango pulps as such or a 2:1 mixture of pulp and syrup and the pH measured. The data are shown in Table 1. The pH of *Raspuri* mango pulp is generally low and round about 3.50. The pH of *Badami* mango pulp varies considerably from about 3.8 to 4.9. On adding acid, the fall in pH is steep in the beginning, but becomes less steep in the later stages. In the case of *Raspuri* mango pulp in combination with half its weight of syrup of 35° or 50° Brix, the fall in pH is from 3.60 to about 2.95, when the added acidity is 1.0 per cent, but in the case of pulp alone, the fall in pH is from 3.60 to 3.20 only. This is accounted for by the fact that the extra acid added in the case of pulp and syrup mixture to bring the syrup also to the same level of acidity as the pulp has no buffer action to counteract as in the case of pulp alone. Similar changes were noticed in the case of the *Badami* mango pulp also.

Addition of acid to canned Badami mangoes. Data regarding the changes in pH of canned *Badami* mangoes as a result of addition of different amounts of citric acid to the syrup, at the end of 2½ hours, one week and 13 months after canning are shown in Tables 2 and 3. The cans were exhausted for 7 minutes at 185-190°F., sealed, processed for 20 minutes in boiling water and cooled quickly in cold water. They were stored at 24-30°C. for different periods.

Soon after canning, the pH of the cut-out syrup is 4.0 when the acidity of the syrup used is 0.31%. The acidity is 0.27% only, the small decrease being due to diffusion of the acid between the fruit and the syrup. When the acidity of the original syrup is 0.46%, the pH of the cut-out syrup is 3.95 and when the acidity is 0.60, the pH is 3.65. At this latter stage, the canned product tastes acidic, indicating that it is not desirable to increase the acidity beyond about 0.5%. Even at the 0.3% level of acidity of the syrup, the pH being 4.00, it may be possible to process *Badami* mangoes in boiling water with this acidity. As a general method,

TABLE 2

pH of Badami mangoes canned[1] in heavy syrup, 2½ hours after canning.

Expt. No.	Acidity in orig. syrup as citric	Drained weight of fruit	Brix of final syrup at 20°C.	Acidity of final syrup, as citric	pH of final syrup	Change in acidity of the syrup	Description of taste
	%	%	°Brix	%		%	
M65	0.00	55.4	32.0	0.12	4.45	0.12	Sweet
M66	0.08	55.2	31.5	0.14	4.45	0.06	Sweet
M67	0.16	55.7	32.0	0.17	4.40	0.01	Sweet
M68	0.31	55.2	32.5	0.27	4.00	−0.04	Sweet
M69	0.45	54.9	33.5	0.27	3.95	−0.19	Sweet
M70	0.60	55.7	32.5	0.38	3.65	−0.22	Slightly acidic
M71	0.75	47.8	32.5	0.65	3.45	−0.10	Acidic
M72	0.90	49.3	32.5	0.75	3.30	−0.15	Acidic
M73	1.04	51.8	32.5	0.79	3.25	−0.25	Markedly acidic

[1] Mango slices, 227 g.; syrup of 50°Brix, 128 g.; plain jam size can.

TABLE 3

pH of Badami mangoes canned[1] in heavy syrup after storage for one week and after storage for 13 months at 24°-30°C.

Expt. No.	Acidity in original syrup	Drained weight of fruit	Brix of final syrup at 20°C.	Acidity of final syrup	pH of final syrup	Change in acidity of the syrup	Description of taste
	%	%	°C	%		%	
M32	0.00	61.6	29.0	0.22	4.35	+0.22[2]	Sweet
		62.1	28.6	0.24	4.20	+0.24	
M33	0.08	59.4	29.0	0.20	4.35	+0.12	Good
		62.4	28.3	0.40	3.95	+0.32	
M34	0.16	67.0	27.0	0.39	3.95	+0.23	Good
		62.3	29.3	0.34	3.85	+0.18	
M35	0.31	62.3	27.5	0.43	3.80	+0.13	Good
		61.8	28.8	0.45	3.68	+0.14	
M36	0.46	62.7	28.0	0.54	3.70	+0.08	Very slightly acidic
		62.1	27.8	0.41	3.65	−0.05	
M37	0.60	60.8	29.0	0.49	3.65	−0.12	
		62.4	27.8	0.47	3.65	−0.24	
M38	0.75	59.1	29.0	0.52	3.65	−0.23	
		62.9	28.1	0.53	3.45	−0.23	
M39	0.90	60.7	28.5	0.53	3.65	−0.37	Slightly acidic
		61.3	29.4	0.54	3.48	−0.36	
M40	1.04	59.4	29.0	0.51	3.65	−0.53	Slightly acidic
		55.4	30.8	0.56	3.32	−0.48	

[1] Mango slices, 227 g.; syrup of 50°Brix, 128 g.; plain jam size can.
[2] NOTE: The data above the line are after one week's storage and below the line after 13 months' storage.

however, the addition of acid to the extent of about 0.5% to syrup of about 50° Brix may be recommended for the safe processing of *Badami* mangoes. The acidity of the syrup has not stabilized itself at the end of 8 days. At the end of 13 months, the changes in acidity are more regular and correspond to the increasing amount of acid added to the syrup. Similar observations hold good in the case of pH also.

Addition of acid to canned Raspuri mangoes. Changes in acidity and pH of the syrup in the case of *Raspuri* mangoes at the end of 14 and 17 months of storage at 24-30°C., are shown in Table 4. With increasing quantities of citric acid ranging from 0.0 to 1.04% added to the syrup, the pH of the cut-out syrup decreased gradually from 4.50 to 3.65, the values being 3.90 and 4.05 when the added acidity was 0.31 and 0.46%, respectively. Somewhere between these two levels of added acid, there was equilibrium between the added acid and the acid in the fruit. Beyond this acidity, the percentage acidity of the cut-out syrup was less than that of the syrup used for canning. The pH values at the end of 14 and 17 months of storage were almost the same.

Addition of acid to canned Neelum mangoes. In the case of *Neelum* mangoes, the shift in the change of percentage acidity of the cut-out syrup changed from positive to negative, when the acidity of the original syrup

TABLE 4

pH of Raspuri mangoes canned[1] in light syrup after storage for 14 and for 17 months at 24°-30°C.

Expt. No.	Acidity in original syrup	Drained weight of fruit	Brix of final syrup at 20°C.	Acidity of final syrup	pH of final syrup	Change in acidity of the syrup	Description of taste
	%	%	°Brix	%		%	
M43	0.00	59.8	23.9	0.14	4.50	+0.14[2]	Very sweet
		56.5	23.4	0.16	4.45	+0.16	
M44	0.08	59.6	23.9	0.21	4.25	+0.13	Good
		57.5	23.4	0.29	4.03	+0.21	
M45	0.16	60.2	23.9	0.21	4.35	+0.05	Good
		61.8	23.4	0.28	4.20	+0.12	
M46	0.31	58.6	23.4	0.34	3.90	+0.03	Good
		61.6	23.4	0.31	4.08	+0.00	
M47	0.46	59.2	23.4	0.30	4.05	−0.16	Good
		58.4	23.4	0.34	3.98	−0.12	
M48	0.60	57.9	23.4	0.36	3.85	−0.25	Very slightly sour
		58.4	22.4	0.38	3.88	−0.22	
M49	0.70	60.0	22.9	0.36	3.90	−0.39	Slightly sour
		57.9	23.4	0.42	3.78	−0.33	
M50	0.90	57.5	22.9	0.48	3.65	−0.42	Sour
		59.7	24.4	0.43	3.58	−0.47	
M51	1.04	58.6	23.4	0.52	3.65	−0.52	Sour
		58.2	23.4	0.50	3.65	−0.54	

[1] Mango slices, 227 g.; syrup of 35°Brix, 128 g.; plain jam size can.
[2] The data above and below the line are after 14 and 17 months' storage respectively.

was increased from 0.21 to 0.31%. On account of the fairly low pH of this fruit (pH 4.3) the addition of about 0.10-0.20% of acid will be sufficient to lower the pH to about 4.0.

Utilization of Bangalora and Raspuri mangoes for canning other fruits having a high pH. *Bangalora* mangoes are highly acidic and when canned alone in syrup of about 50°Brix, the pH of the cut-out syrup will be as low as 3.22. Similarly, in the case of some *Raspuri* mangoes also, the pH is fairly low (3.60). To study to what extent it is feasible to use these fruits to can along with other fruits like jack fruit, banana, *Coorg* orange segments, etc., in different proportions, a series of canning trials were carried out. Both acidified and non-acidified syrups were used. The results are given in Table 5. *Bangalora* mango, orange and jack fruit bulbs can be safely canned in the ratio of 2:1 to 3:1 as the pH of the cut-out syrup is as low as 3.80-3.85, although the pH of the jack fruit pulp is as high as about 5.20. Acidic *Raspuri* mangoes also can be employed to can jack fruit bulbs, *Salem* and *Nanjangud* bananas and *Coorg* orange segments in the ratio of about 2:1 to 3:1. The pH of the syrup, in these cases is, however, quite near to 4.20-4.40 and the margin of safety is comparatively low. The addition of about 0.5% acid to the syrup will bring the pH well below the safe limit. *Badami* mangoes can be safely canned along with

TABLE 5

pH of Bangalora, Raspuri, and Badami mangoes canned with fruits of different acidities. (Storage period 15 months at 24°-30°C.)

Expt. No.	Fruits canned	Original syrup		Cut out syrup			Description of taste
		Brix	Acidity	Brix	Acidity	pH	
		°Brix	%	°Brix	%		
	Bangalora mango						
M 91	*Bangalora* mango only	50	0.0	27.5	0.51	3.22	Mango slices yellow; slightly soft
M 92	*Bangalora* mango + jack fruit (2:1)	50	0.0	29.4	0.42	3.85	Jack fruit rather soft; otherwise a good product
M 93	*Bangalora* mango + jack fruit (2:1)	50	0.5	30.4	0.61	3.62	The same as above
M 116	*Bangalora* mango + jack fruit (3:1)	50	0.0	28.4	0.40	3.80	The same as above
M 96	*Bangalora* mango + pineapple (3:1)	50	0.0	25.3	0.61	3.28	Canned fruit rather acidic
	Raspuri mango						
M 53	*Raspuri* mango + pineapple (1:1)	50	0.0	29.9	0.34	3.98	Good blend; a good product
M 54	*Raspuri* mango + pineapple (1:1)	50	0.5	29.9	0.51	3.58	The same
M 63	*Raspuri* mango + pineapple (2:1)	50	0.0	29.9	0.20	4.38	The same
M 61	*Raspuri* mango + pineapple (2:1)	50	0.5	22.3	0.42	3.74	The same
M 78	*Raspuri* mango + *Nanjangud* banana (2:1)	50	0.0	29.4	0.25	4.30	Good blend; good product
M 79	*Raspuri* mango + *Nanjangud* banana (2:1)	50	0.5	29.9	0.42	3.82	The same
M 80	*Raspuri* mango + Salem banana (2:1)	50	0.0	27.3	0.38	3.84	Banana slices slightly hard and starchy
M 81	*Raspuri* mango + Salem banana (2:1)	50	0.5	26.8	0.43	3.85	The same
M 86	*Raspuri* mango + Coorg orange segments (3:1)	50	0.0	27.3	0.24	4.18	Fruits in good condition; a good product
M 87	*Raspuri* mango + Coorg orange segments (3:1)	50	0.5	28.4	0.40	3.72	The same
J 87	*Raspuri* mango + jack fruit (1:2)	50	0.5	31.4	0.32	4.42	Fruits in good condition; characteristic jack fruit flavour. Rather sweet.
J 89	*Raspuri* mango + jack fruit (1:2)	50	0.5	33.9	0.40	4.12	
J 89	*Raspuri* mango + jack fruit (1:2)	35	0.15	28.4	0.21	4.71	Highly sweet
	Badami mango						
M 56	*Badami* mango + pineapple (2:1)	50	0.0	29.9	0.39	3.80	Both fruits in good condtion; good product
M 57	*Badami* mango + pineapple (2:1)	50	0.5	28.4	0.60	3.45	The same
M 58	*Badami* mango + pineapple (2:1)	35	0.0	27.3	0.38	3.80	The same
M 59	*Badami* mango only	50	0.0	29.4	0.33	3.95	A very good canned product; bright orange yellow mango slices
M 60	*Badami* mango only	50	0.5	27.3	0.46	3.62	The same
M 74	*Badami* mango + *Nanjangud* banana (2:1)	50	0.0	29.9	0.25	4.25	Banana slices slightly hard in texture
M 75	*Badami* mango + *Nanjangud* banana (2:1)	50	0.5	28.4	0.43	3.80	The same
M 76	*Badami* mango + *Salem* banana (2:1)	50	0.0	29.9	0.23	4.28	A good canned product
M 77	*Badami* mango + *Salem* banana (2:1)	50	0.5	29.4	0.45	3.78	The same
M 84	*Badami* mango + Coorg orange segments (3:1)	50	0.0	29.4	0.32	4.20	Mango slices in good condition. Orange segments slightly broken; otherwise a good canned product.
M 85	*Badami* mango + Coorg orange segments (3:1)	50	0.5	28.4	0.34	3.80	The same

pineapples in syrup of 35° or 50° Brix without the addition of any extra acid as the pH of the syrup will be about 3.80. For canning these mangoes with other fruits like banana and orange segments, the addition of about 0.5% acid to the syrup is desirable to render the ordinary processing quite safe. The different combinations tried in these experiments were found to be compatible and the canned product (fruit salad type) was quite acceptable. The taste and aroma of jack fruit were mellowed down, while those of banana and orange were enhanced.

SUMMARY AND CONCLUSION

The pH of *Raspuri* mango pulp is generally low (about 3.50) while that of *Badami* mango pulp varies from 3.80 to 4.50.

In the case of *Raspuri* mango pulp in combination with half its weight of syrup, the pH decreases from 3.60 to 2.95 when 1% acid is added. Similar changes occur in the case of *Badami* mango pulp also.

For the safe processing of *Badami* mangoes, it is desirable to add 0.3-0.5% acid to the syrup. In the case of *Raspuri* and *Neelum* mangoes, the addition of acid is not always necessary.

Bangalora mangoes having a low pH (about 3.20) can be employed to lower sufficiently the pH of fruits like jack fruit, banana, and orange segments, which have ordinarily a high pH of 4.80-5.20. *Badami* mangoes can be canned safely with a more acid fruit like pineapple by employing ordinary processing in boiling water. The canned mixed fruits are quite acceptable.

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THE FORMATION AND DECOMPOSITION OF GREEN PIGMENT IN CRUSHED GARLIC TISSUE

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In the preparation of garlic puree, garlic juices, and garlic powders, for use as condiments, discoloration and undesirable changes in flavor have been ascribed to enzyme catalyzed oxidations *(3)*. The desirable flavoring constituents of garlic, however, are formed from a naturally occurring flavorless precursor, alliin, an unsaturated sulfur-containing amino acid, by the action of the tissue enzyme, alliinase. Alliinase rapidly converts alliin into the mixture of constituents characteristic of garlic flavors. In garlic this change occurs only when the tissue is crushed, and the intact garlic bulb is free from garlic flavor. Alliinase has a wide optimal pH range (pH 5-8) but a sharp optimum temperature (37°C., 98.6°F.). Above this optimum temperature its activity decreases rapidly and it is inactivated at temperatures above 50°C. (140°F.) *(11)*. The heat treatment of garlic purees must be conducted under conditions which do not interfere with the enzymatic development of garlic flavor. The darkening of extracts of garlic containing 10% added salt or 2.5% acetic acid, commonly observed, was ascribed by Sugihara and Cruess *(12)* to the activity of an "oxidase." The peroxidase in partially purified preparations was found to have an optimum pH of 5.05-5.20 and was inactivated by heating at 90°C. (194°F.) for 5 minutes at pH 7.0. Garlic extracts prepared by macerating garlic cloves, mixing with an equal volume of 50 grain vinegar or with 10% by weight of salt, when heated to 90-100°C. (194-212°F.) for a period of about 5 minutes before pressing, were found to remain light in color and of normal, fresh garlic odor for over a year *(12)*. Pectolytic enzymes *(7)* and bacteriostatic and bactericidal activity of garlic *(2)*, also, have been investigated.

Until recently commercial garlic tissue preparations (garlic purees containing added salt or vinegar, garlic juices, and garlic oils) did not retain the desired flavor of freshly prepared garlic. A satisfactory garlic puree was developed by crushing the whole garlic bulb, separating skins and other undesirable tissue by passage through a pulper, heating the resulting puree, and additional juice recovered by pressing the pulp discharged in the pulper, in a continuous tubular heat exchanger and adding 10% salt to the resulting puree. During the development of this process, the formation of an intense green color was observed when the heated purees were stored at room temperature *(14)*. This pigment was found to be readily soluble in acetone but only sparingly soluble in hexane, petroleum ether, or other solvents and did not behave like chlorophyll or related porphyrin pigments *(4)*.

The formation in plant tissue of a green pigment from a colorless precursor is of importance not only practically but also scientifically. Protochlorophyll, the colorless precursor of chlorophyll, has been investi-

gated *(5, 9)*, and a porphyrin pigment has been isolated from photosensitive non-chlorophyllous plant tissue *(13)*. Bate-Smith *(1)* recently reported that extracts (with 1% hydrochloric acid in methanol) of certain yellow or cream colored flowers became distinctly green after standing for an hour or two. He observed, also, transient green coloration with leuco anthocyanin preparations shaken with aqueous alkali. When definite green coloration was present, a residual yellow carotenoid and an unstable bright blue pigment scarcely soluble in petroleum ether were detected *(1)*. Several of the factors influencing the formation of green pigment in garlic tissue macerates (pH, temperature, and time of heating, salt and sulfite content) were investigated by Sano *(8)* and some observations on the nature of this pigment were made. The results of these investigations are presented here.

EXPERIMENTAL METHODS

Bulbs of the garlic plant *Allium sativum L* were obtained from commercial market and other sources.[a] These represented both early and late California summer grown garlic and Mexican garlic. The garlic bulbs thus obtained were stored at 0°C. (32°F.) until used.

Before use, the garlic bulbs were separated into the individual sound cloves. The cloves were then carefully peeled with a stainless steel paring knife, cut in half and the inner green shoots were removed. The halved cloves were carefully examined and those with green portions remaining were separated. The green portions of tissue were then cut off and the non-green portions returned to the main batch.

One hundred and fifty grams of the garlic prepared as above were added to a Waring blender, together with 50 ml. of distilled water to facilitate blending. The mixture was blended until a smooth puree was obtained (usually for 3 minutes). The pH of the puree was 5.6. Where necessary, the pH was adjusted by addition of 0.5 N sodium hydroxide or hydrochloric acid. Twenty-gram portions were weighed into 50 × 150 mm. test tubes. The samples were stored at room temperature (22°C., 70°F.) until they were extracted. When heat was applied the samples were first held in boiling water and stirred constantly to minimize the come up time. When the desired temperature (in the range of 50-100°C., 122-212°F.) had been reached the test tubes were transferred to a constant temperature water bath. Thus when the sample reached a temperature of 50°C., it was transferred to a 50°C. water bath and kept in the water bath for 3 minutes. It was then removed at the end of 3 minutes and cooled to 22°C. with cold running (14°C.) tap water while being stirred. The cooled samples were held for 24 hours prior to extraction.

In preparing the extracts for color measurements, weighed amounts of garlic puree (20 g.) were transferred to the Waring blender and measured amounts (50 ml.) of C.P. acetone were added. The sample was then blended for 3 minutes and the resulting mixture was filtered through Whatman No. 1 filter paper. The filtrate was then made up to 50 ml. with acetone and transferred to a Klett colorimeter tube. Readings were made with the No. 42, 54, 60, and 66 filters. The Klett-Summerson photocolorimeter was adjusted to zero with acetone solution of the same concentration as in the unknown.

In preliminary investigations, garlic bulbs purchased on the local market and those obtained from Davis were used. Various solvents were tested to determine the solubility characteristics of the pigment. Ethyl acetate, benzene, absolute methyl alcohol, n-butyl alcohol, 95% ethyl alcohol, isobutyl alcohol, carbon tetrachloride, isoamylacetate, n-butyl chloride, toluene, xylene, 1:1 methyl alcohol-benzene, and diethylpthalate were used. Assuming that this pigment may be a complex, various reagents were used to attempt to break this complex. They were: sodium sulfate, sodium chloride, sodium hydroxide,

[a] The cooperation of the Department of Vegetable Crops, University of California, Davis, and the Enchantment Food Products Company of Sebastopol, California, in supplying garlic is gratefully acknowledged.

hydrochloric acid, acetone, dioxane and saponin. Also 1:1 magnesia-silica, silica, talc, and solka floc chromatographic columns were used in attempts to separate the pigment. The absorption spectra of the acetone extracts of the green and brown pigment were measured in a Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

Characteristics of pigment or pigments formed. The formation of a green pigment from a colorless precursor resembles the formation of chlorophyll from protochlorophyll. The acetone extracts of the uncrushed garlic, however, had little or no specific absorption, unlike that characterizing protochlorophyll *(5)*. The absorption spectrum of the acetone extract of the greened crushed garlic, plotted as optical density versus wave length on semi-log paper, Figure 1, unlike chlorophyll, has a minimum absorption

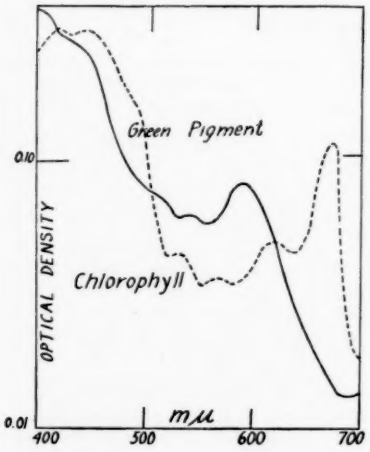

Figure 1. Absorption spectrum of the acetone extract of greened garlic in comparison with that of chlorophyll.

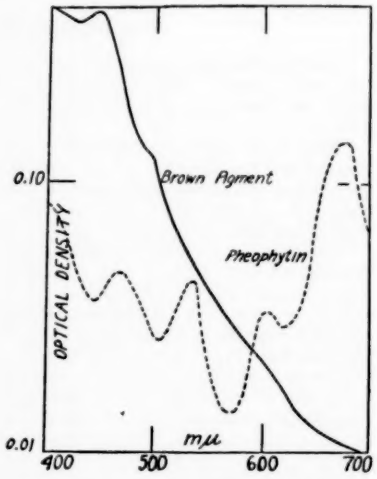

Figure 2. Absorption spectrum of the acetone extract of brown pigment formed from previously greened garlic in comparison with that of pheophytin.

at 560 mμ and a maximum at 585 mμ. In 80% aqueous acetone, chlorophyll a has absorption maxima at 660 mμ and 434 mμ, and chlorophyll b at 645 mμ and 455 mμ *(6)*. The green pigment formed in crushed garlic tissue yields a clear solution in 80% acetone-water. On standing, the solution becomes turbid, an amorphous precipitate forms, and the green pigment slowly becomes yellowish or brown. The browning occurs more rapidly at high pH values rather than low pH values. The yellow-brown extract, Figure 2, has a maximum at 440 mμ, unlike pheophytin with maxima at 460, 535, 600, and 665 mμ.

Partial fractionation was obtained with petroleum ether. With the other solvents there was no fractionation. Separation of the pigment by chromatographic and other reagents was unsuccessful also. The copper and iron content of crude pigment was not related to color intensity.

With an increase in acetone concentration precipitation of the green pigment occurs and a clear acetone solution results, unlike that of the chlorophyll-protein compound of green leaves *(9)*.

It was observed that the late summer garlic will form the green pigment much more readily than the early summer and Mexican garlic at the natural pH of 5.6.

The green pigment or pigments will not form if the individual cloves are cut in half or quartered. The garlic tissue must be thoroughly macerated for pigment formation. If large pieces of cloves are present in the garlic puree, they will remain uncolored even after the puree has greened. This is similar to the conditions necessary for formation of garlic oil *(11)*.

Whether one or more than one green pigment is formed is not known. The pH of the puree, however, influences the tint. At pH of 5 and above the green color visually differed from that formed at pH 4 and below, which appeared bluer. This may be due either to formation of different pigment mixtures or to the effect of pH on the pigment formed. Phenolase and peroxidase activity were not found necessary for color formation, since greening occurred whether these enzymes were present or not. The green extracts obtained from garlic tissue heated sufficiently to completely inactivate both peroxidase and phenolase gradually cloud on standing, form an amorphous sediment and lessen in green tint. Extracts of tissues heated to 100°C. (212°F.) for 3 minutes, considerably above the temperature required to inactivate peroxidase, remain clear and do not produce sediment. If the clouding is due to denaturation of proteins or other colloids this must occur more extensively at temperatures below 100°C.

Rate of pigment formation at room temperature. Commercially grown late summer garlic from Salinas Valley was macerated as described previously. The purees, at pH 6, were stored for 0 to 288 hours at room temperature (22°C., 70°F.) before extraction with acetone. The data obtained are shown in Table 1 and Figure 3. Figure 3 shows the changes in color as measured by changes in the absorption of light at several regions of the spectrum. The colorimeter readings with Nos. 54, 60, and 66 filters rapidly increased to a maximum in 24 hours. After 72 hours the puree began to brown at the surface layers in contact with air and this browning increased with time and extended into the lower layers. After 144 hours (6 days)

TABLE 1

Rate of pigment formation in garlic purees stored at room temperature.

Time stored hours	Colorimeter reading			
	Filter used			
	No. 42	No. 54	No. 60	No. 66
0	34	22	13	15
4	38	29	17	14
24	113	47	38	22
48	107	39	26	15
72	118	40	26	18
96	138	45	27	20
120	128	40	21	16
144	158	46	22	13
168	149	42	19	15
288	144	42	20	19

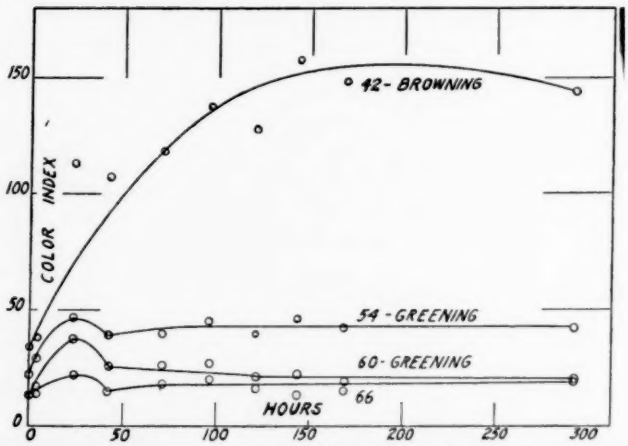

Figure 3. Effect of storage at 22°C. and pH 6 on color formation in macerated garlic tissue. The colorimeter reading with filters shown on the individual graphs is plotted against time of storage.

storage the puree was more brown than green and at 288 hours (12 days) it was brown on top and yellow underneath. The rate of browning is indicated by the increase in colorimeter reading with the No. 42 filter (approximate spectral range of 400-465 mμ) in the region where the brown garlic pigment exhibits maximum light absorption. With this filter, the colorimeter readings increased from the start of storage and continued to increase after maximum greening had been attained. After about 200 hours browning reached its maximum and then began to decrease.

Effect of pH and temperature of heating. It was observed under commercial conditions *(14)* that the greening of garlic puree occurs more rapidly at certain stages of heating in a continuous tubular heat exchanger. Therefore, the effect of temperature and pH on color formation in garlic purees was determined. For this purpose garlic puree was adjusted to pH of 2 to 8, before heating, heated to and held for 3 minutes at 22, 50, 60, 70, 80, 90, and 100°C. It was then cooled to room temperature and stored at room temperature for 24 hours before extracting with acetone. The data obtained are shown in Table 2 and in part in Figures 4 and 5. The purees were observed before extraction with acetone but time did not permit color evaluation by reflectance color meter. The visual observations of purees at various pH values are summarized as follows:

At pH 8 the ground garlic puree, before heating, showed almost no green color and appeared straw in color. With the increase in temperature the puree became a deeper straw color. The sample heated to 100°C. was almost a light brown.

At pH 7, the puree appeared green at the lower temperatures up to 60°C. Above 60°C. the puree was yellow-green in color and at 100°C. it was a yellow-brown.

At pH 6, the puree at lower temperatures was green in color while at 90°C. it was yellow in color and at 100°C. was a yellowish-brown.

TABLE 2

Effect of pH and temperature on color formation.

pH	Filter No.	Temperature in degrees Centigrade						
		22	50	60	70	80	90	100
		Colorimeter reading						
2	42	89	89	89	95	104	153	233
	54	25	23	23	22	24	36	54
	60	16	16	13	15	16	31	46
	66	16	14	15	14	14	19	25
3	42	100	99	94	101	134	138	139
	54	39	40	34	40	44	55	40
	60	20	20	17	26	32	52	25
	66	22	19	18	19	23	29	22
4	42	80	86	86	92	100	131	159
	54	39	46	42	49	46	55	49
	60	34	45	45	57	47	50	38
	66	25	28	28	33	28	27	24
5	42	68	70	65	65	64	66	68
	54	34	40	32	31	29	27	22
	60	36	46	32	24	19	11	7
	66	24	26	21	18	15	14	14
6	42	69	73	63	71	57	53	67
	54	33	33	30	31	28	23	27
	60	22	21	19	18	12	8	11
	66	16	15	13	16	11	10	10
7	42	53	55	55	56	56	55	65
	54	31	26	28	22	26	20	24
	60	17	11	13	7	9	5	7
	66	16	16	14	12	12	10	9
8	42	48	43	42	38	44	53	47
	54	28	30	25	25	24	28	22
	60	18	17	13	12	11	12	9
	66	16	17	16	15	15	16	14

At pH 5, up to 90°C., the samples were green with a slight blue tint. At 90°C. the puree was yellow-green and at 100°C. it appeared to be a yellow-brown.

At pH 4, the puree was a blue-green in color up to 100°C. where it became green.

At pH 3, the samples up to 60°C. did not change in color, but at 70 and 80°C. there was a slight blue-green color which was stronger at 90°C. and which turned to a green color at 100°C.

At pH 2, the samples heated from 22 to 70°C. did not exhibit green color formation but at 80 and 90°C. they became slightly bluish-green in color and at 100°C. they became green.

The effect of pH and temperature of heating on green pigment formation as measured by colorimeter readings made with No. 60 filter (approximate spectral range of 580-640 mμ), in the region where the green garlic pigment exhibits maximum light absorption in acetone extracts, is shown in Figure 4. The data plotted in Figure 4 clearly indicate that at 22 and

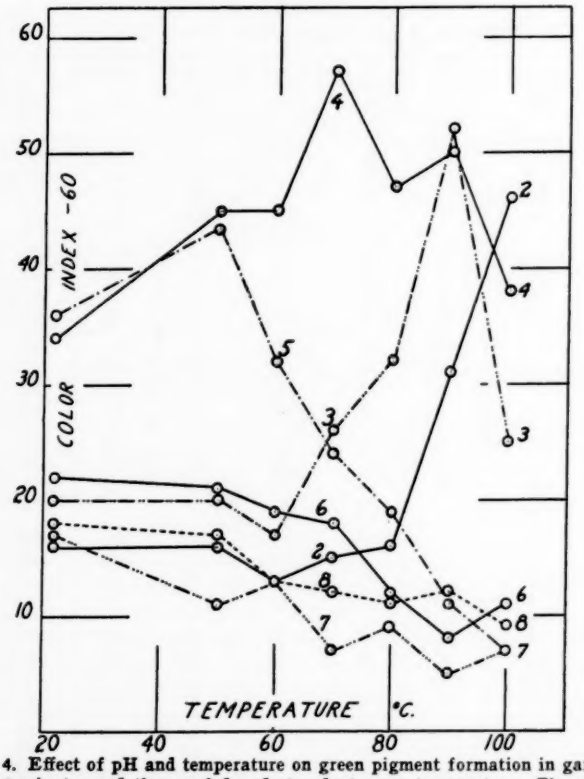

Figure 4. Effect of pH and temperature on green pigment formation in garlic purees heated for 3 minutes and then cooled and stored at room temperature. The colorimeter readings with No. 60 filter of acetone extracts of purees adjusted to pH values shown on graphs and heated for 3 minutes at various temperatures are plotted against temperature.

50°C., greening is greatest at pH 4 and 5 and is at a maximum at 70° at pH 4, being followed closely in purees at pH 3 heated to 90°C. and pH 2 heated to 100°C. At pH values lower than pH 5, greening was more intense at higher temperatures and at pH values greater than pH 5 greening decreased in intensity as temperature increased.

The browning of these purees is shown in Figure 5. At pH range of 2 to 4, browning was higher at lower temperatures than at pH range of 5 to 6, and increased more rapidly, particularly at higher temperatures, with temperature. Maximum browning occurred at pH 2 at 100°C., being followed by that at pH 4 and 3 at 100°C. At pH 2 and pH 4 browning increased very rapidly in the temperature range of 80-100°C.; at pH 3 it increased rapidly in the range of 70-80°C. and then remained essentially constant. At pH 5-8, browning was less at lower temperatures than at lower pH values, and either remained constant (pH 6) or decreased in the range of 20 to 90°C. At 100°C., browning was least at pH 8 and highest at pH 2.

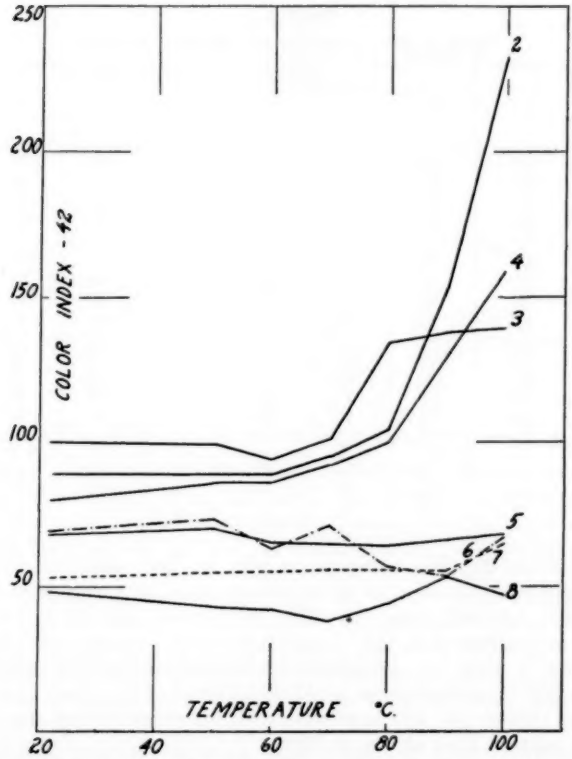

Figure 5. Effect of temperature of heating on browning of garlic at various pH values. Colorimeter readings with No. 42 filters at pH values shown on the graphs and heated for 3 minutes at various temperatures are plotted against temperature.

Effect of time of heating on color formation at pH 4. To determine how the time of holding affects color formation, garlic purees at pH 4 were heated at 60, 70, 80, and 90°C. for 0 to 10 minutes. After cooling and storage at room temperature for 24 hours, they were extracted. The results obtained are shown in Table 3. The data indicate that greening (No. 60 filter) reached a maximum in 8 minutes at 60°C., 2 minutes at 70°C., 1 minute at 80°C. and when brought to temperature at 90°C. As heating increased, the color formation decreased. Browning (No. 42 filter), on the other hand, generally increased in intensity as the period of heating increased.

Effect of sodium chloride. Sodium chloride was added to garlic puree at pH 4 at two levels, 10 and 20%. The purees were stored unheated (22°C.) and after heating for 3 minutes at 70 and 100°C. The results obtained are shown in Table 4.

The data indicate that at 22°C. the addition of sodium chloride slightly decreased the amount of green pigment formed, the decrease in colorimeter

TABLE 3

Effect of time of heating on pigment formation.

Tmperature	Filter No.	Time held at temperature in minutes						
		0	1	2	4	6	8	10
		Color reading						
60°C.	42	86	89	94	94	99	106	104
	54	31	39	36	40	36	45	37
	60	47	55	55	59	60	63	60
	66	28	30	32	32	34	31	32
70°C.	42	91	96	104	104	108	118	117
	54	41	36	48	35	47	41	46
	60	56	60	71	60	63	64	60
	66	32	34	34	32	34	35	32
80°C.	42	100	103	104	104	114	125	123
	54	42	48	40	47	40	46	38
	60	66	71	63	63	56	52	44
	66	36	37	35	33	31	30	29
90°C.	42	110	112	111	120	113	116	125
	54	41	44	34	38	29	36	28
	60	72	63	52	44	34	37	28
	66	36	32	26	25	21	21	17

reading (Filter No. 60) being about 10% at 10% salt and 20% at 20% salt. In purees preheated to 70°C., the decrease in green pigment formation at 10% salt level was only 10% but at 20% salt level it was almost 40%. In purees heated to 100°C., green pigment formation was not affected by salt at 10% level but reduced by about 25% at the 20% salt level. The green at 100°C. appeared to be of a different shade than that at 70°C. Browning (Filter No. 42) increased with temperature but was greater at 10% salt content than at 0% or 20%.

Effect of sulfur dioxide. Sulfur dioxide was added as sodium bisulfite at levels of 500, 1000, 2000, and 5000 p.p.m. For comparison, a control

TABLE 4

Effect of sodium chloride on color formation.

Filter No.	Temperature °C.	Per cent sodium chloride added		
		0	10	20
		Colorimeter reading		
42	22	83	108	118
54	22	24	29	30
60	22	43	39	35
66	22	27	27	24
42	70	104	117	103
54	70	48	37	31
60	70	71	64	44
66	70	34	32	26
42	100	159	179	151
54	100	49	41	34
60	100	43	45	33
66	100	24	26	19

TABLE 5

Effect of sulfur dioxide on color formation.

Filter No.	Temperature °C.	Concentration of sulfur dioxide in p.p.m.				
		0	500	1000	2000	5000
		Colorimeter reading				
42	22	88	83	83	81	72
54	22	44	42	40	38	30
60	22	63	57	58	52	40
66	22	42	41	41	37	32
42	70	103	121	118	100	79
54	70	46	63	62	57	57
60	70	70	102	115	103	54
66	70	32	56	64	57	37
42	100	140	114	144	137	110
54	100	49	40	49	46	36
60	100	44	45	49	47	40
66	100	30	32	30	29	28

sample without any sodium bisulfite was prepared. These were heated to 70°C. and 100°C., as well as unheated. The results obtained are shown in Table 5.

At room temperature (22°C.) the addition of sodium bisulfite decreased the amount of green pigment formed, but did not inhibit it completely. At 70°C., greening increased with increase in SO_2 content up to 1000 p.p.m. and then decreased; browning, on the other hand, was at a maximum at 500 p.p.m. At 100°C., the sulfur dioxide content affected greening only slightly but there was an indication of maximum greening at 1000 p.p.m.; browning on the other hand, was higher at 1000 and 2000 p.p.m. of SO_2 than at 0 or 5000.

Stability of acetone extracts. The originally clear acetone extracts obtained from purees adjusted to various pH values and heated to various temperatures were stored to observe changes in appearance. Colorimeter readings were made at 0, 1, 3, 5, 15, 30, and 50 days with all four filters. Typical of the data obtained are those shown in Figures 6 and 7 and Table 6. Only the colorimeter readings with No. 42 filter varied appreciably with time of storage at room temperature and these indicate gradual browning.

The increase in brown pigment formation is shown in Table 6 as increase in colorimeter reading after 50 days storage of the acetone extracts. In

TABLE 6

Effect of storage of acetone extracts.

pH	Original temperature at which heated						
	22	50	60	70	80	90	100
	Increase in colorimeter reading with filter No. 42 after 50 days						
3	335	251	251	246	258	227	101
4	241	262	264	224	227	158	151
5	234	239	213	195	197	190	140
6	331	330	362	299	301	283	221
7	326	385	445	327	272	216	107

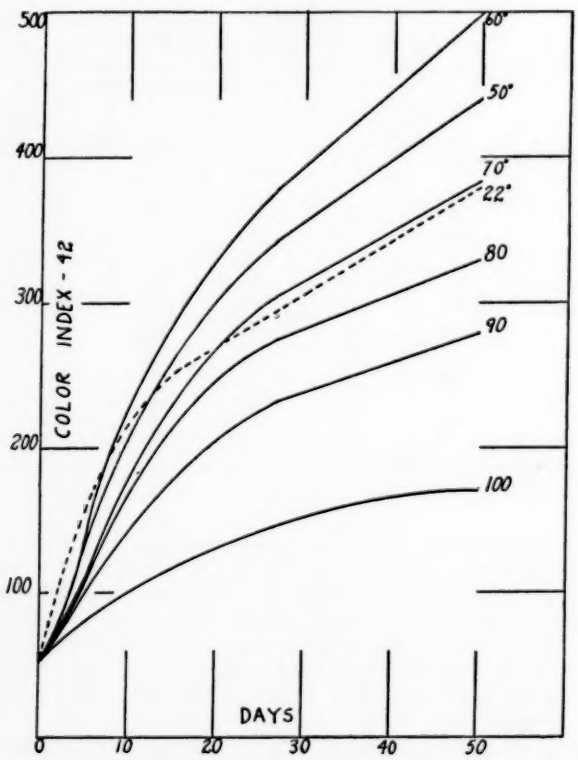

Figure 6. Effect of storage of acetone extracts of garlic puree adjusted to pH 7 and heated to temperatures shown on the graphs.

Figure 6, the browning of the extracts from purees adjusted to pH 7 and heated to various temperatures is plotted in more detail. The rate of browning was faster in extracts of purees heated to 50-70°C. than in the unheated puree or in purees that were heated to 80-100°C. The puree heated to 100°C. at pH 7 gave an extract that browned less slowly and reached a lower level of browning. In Figure 7, the browning of the extracts from puree adjusted to various pH levels and heated to 100°C. is plotted in more detail. The decrease in colorimeter reading in purees at pH 3 and 4 in the first 5 days is due to precipitation of pigment. After this happened browning of the extracts of purees at pH 4 was as fast as in those obtained from purees at pH 6 but the level of pigment formation was greater. Browning was least in extracts prepared from purees at pH 7.

A slight precipitation occurred in all of the samples at pH 4 and below within one day. At pH 5, 6, and 7 precipitation occurred at all the temperatures below 100°C., and only at 100°C. did the sample remain clear. The colorimeter reading with No. 60 filter usually was greater than with the 54 or 66 filters for the greenest sample, at optimum pH and tempera-

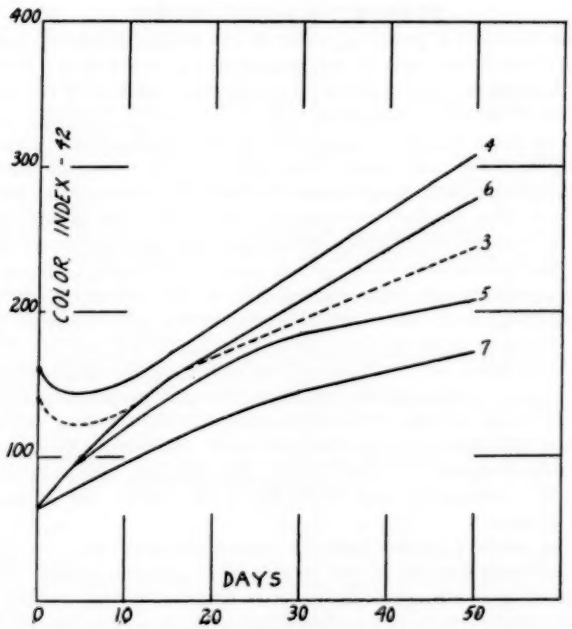

Figure 7. Effect of storage of acetone extracts of garlic puree adjusted to pH values shown on graph and heated to 100°C.

ture. This value, however, drops below that of the 54 filter within one day, but with this drop the solution will still be green. When the 42 filter value reached 150, the solution appeared orange-brown, and this color usually was reached in 15 days.

The results summarized in Table 6 indicate that the most stable extracts were those obtained from purees at pH 3 and 7 heated to 100°C., next that from puree at pH 4 and 5 heated at 100°C. and least stable from puree at pH 6 heated at 100°C. Purees heated to lower temperatures gave extracts that were less stable and the least stable extract was that obtained from unheated puree at pH 3.

The colorimeter readings for acetone extracts of purees at pH 4, heated at 60, 70, and 90°C. for 0 to 10 minutes, were also observed periodically during storage for 30 days at room temperature. Those obtained from purees heated at 60°C. increased in color value from 214 for 0 minutes of heating to 221 for 10 minutes, the maximum increase (238) occurring with purees heated for 6 minutes. Those obtained from purees heated at 70°C., increased in color value from 204 at 0 time to 167 at 10 minutes, the maximum increase (247) occurring with purees heated for 1 minute. Those obtained from purees heated at 80°C., increase in color value from 220 at 0 time to 127 at 10 minutes, the maximum increase (220) occurring with purees heated for 0 time.

SUMMARY AND CONCLUSIONS

The formation of a green pigment in macerated tissue of garlic cloves free of pre-formed chlorophyll was observed and some of the factors influencing its formation were studied. Complete maceration of the clove tissue was necessary; pigment formation did not occur in halved or quartered cloves and in large pieces of garlic tissue in the macerate. The naturally occurring phenolases and peroxidases were not involved in formation of the green pigment, since tissue macerates held at temperatures high enough and for times long enough to inactivate these enzymes became green and the rate and extent of pigment formation was not related to activity of phenolase and peroxidase. The green pigment formed in macerates held at room temperature at their normal pH of 5.6 but optimum conditions for its formation were pH 4 at 70°C. when the puree was heated for 3 minutes. At room temperature (22°C.) and pH 6.0, maximum greening occurred in 24 hours and then the green puree slowly browned, at first, in the upper layers exposed to air, and then throughout, and finally the brown pigment gradually became yellowish and eventually became a light cream color. These changes in pigmentation were influenced by conditions of heating (pH, temperature and time) and storage conditions (pH, and temperature). Browning was more rapid at higher pH values than at lower pH values.

Both the green pigment and the brown pigment were readily soluble in 70-80% acetone but not in any of the other solvents tested. The acetone extract was clear bright green in color at first but became turbid on standing, and an amorphous, light-colored precipitate settled and the solution slowly browned. The temperature and time of heating and pH of the puree before extraction with acetone determined the relative stability of the acetone extract. Precipitation of amorphous material occurred more rapidly in purees heated at pH 4 or below; at pH of 5 to 7 sediment formation was slower and when puree at these pH levels was heated at 100°C., the acetone extract remained clear. The green pigment precipitated from the acetone extracts as the concentration of acetone was increased from 70-80% to 100%, and did not partition from acetone into petroleum ether. The acetone extracts of the green pigment exhibited a general high absorption in the region of 400-450 mμ. The brown pigment in acetone solution exhibited a sharp absorption maximum at 440 mμ and a rapidly decreasing absorption from this maximum to 700 mμ.

The absorption spectra of the crude green and brown acetone extracts were quite different from those of chlorophyll, pheophytin or the olive-green porphyrin pigment found in several non-chlorophyllous plant organs that respond to red light. The conditions of pigment formation and the factors affecting stability of the pigment in acetone extract render it unlikely that the colorless precursor is similar to protochlorophyll or that the pigment complex itself is similar to the chlorophyll-protein complex of green leaves.

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ORGANIC ACIDS IN PEACHES

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For greatest taste satisfaction from either fresh or processed peaches, the fruit must be picked and consumed as near to optimum maturity as possible. Flavor evaluation studies of canned cling peaches by Leonard, Luh, and Hinreiner *(12)* have shown that total titratable acidity in peaches decreases with maturation and that a fairly reliable indication of flavor rating is provided by the ratio of soluble solids to total acid in the raw fruit, when this value is referred to the total acidity. However, the nature of the organic acids in clingstone peaches was not investigated.

Numerous studies of organic acids in freestone peaches have been published. Bigelow and Dunbar *(3)* reported that only malic acid was present in peaches. Nelson *(18)* found citric acid and malic acid present in almost equal proportions in canned peaches. Nuccorini and Cerri *(21)* found varying concentrations of malic, citric, and tartaric acids at different stages of development for both early- and late-ripening fruits. Peynaud *(23, 24)* investigated the malic and citric acid composition of 36 varieties of peaches grown in France.

Recently, Johnson, Foreman, and Mayer *(11)* reported the presence of chlorogenic acid in peaches. Corse *(5)* discovered an isomer of chlorogenic acid, which he called neo-chlorogenic acid, in ethyl acetate extracts of the Elberta and Halford varieties. Anet and Reynolds *(1)* isolated quinic acid from Golden Queen, J. H. Hale, and Blackburn Elberta peaches and indicated that this acid contributes greatly to the total acidity of the fruit. They also reported the presence of mucic and galacturonic acids *(2)* in small amounts in the fresh fruit.

The majority of the published information on organic acids in peaches, except the work by Anet and Reynolds *(1)*, was based either on precipitation of the organic acids as the barium salt in various concentrations of ethyl alcohol, or on esterification of the acids followed by fractional distillation. Under these conditions, separation of one acid from another may not be complete. Furthermore, no attempt was made to correlate acid changes with an objective measurement of maturity.

One of the programs of the Department of Food Technology at Davis is concerned with factors influencing the quality of canned peaches. One phase of this program considers the importance of chemical constituents of the fruit. In this work, the changes in major organic acids during the development of Halford and Peak clingstone and Fay Elberta freestone peaches were investigated by silicic acid column chromatography, using the method of Bulen, Varner, and Burrell *(4)* to determine acids in fruits. The results are correlated with the maturity of the peach as measured by pressure test. The importance of such changes to the quality of canned peaches is discussed.

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EXPERIMENTAL MATERIALS AND METHODS

Clingstone peaches. Halford and Peak clingstone peaches were obtained from a commercial orchard near Yuba City, California. At different stages of development 50-lb. samples were picked at random from a group of 8 trees. The peaches were brought to the pilot plant at Davis and canned within 4 hours after harvest. The same group of trees was used for 1953 and 1954 crops.

Freestone peaches. Fay Elberta freestone peaches were harvested at different stages of maturity in the 1953 season from the University Farm at Davis. Two fruits of about the same size and color were picked from each of 40 trees. During the 1954 season 10 fruits from each of 10 trees were selected at each picking.

Canning. The samples were sorted to remove specimens smaller than 2¼ inches or larger than 3 inches in diameter. The peaches were selected and sorted, ground skin color serving as a criterion of maturity. They were washed in cold water and then allowed to drain to remove the moisture adhering to the surface. After they were halved and pitted, 18 ± 0.1 oz. of the unpeeled fruit and 11 ± 0.1 oz. of distilled water were put into No. 2½ plain tin cans. The cans were sealed by a mechanical vacuum closing machine under 15 inches of vacuum, cooked in boiling water for 30 minutes, and then cooled to room temperature in running water. The cans were stored at room temperature until analyzed during the following winter months.

Analysis of the fresh fruit. Ten representative peaches of comparable skin color from each lot were used for the investigation of physical and chemical properties. The fresh samples were analyzed on the day of their arrival at Davis. Skin color was determined by reference to standard plates in Maerz and Paul's *Dictionary of Color (16)* under a standard light source. Pressure-test determinations were made on the peeled cheek area of the fruit, using the Magness-Taylor pressure tester with a 5/16-inch plunger, to a depth of 5/16 inch. A second pressure tester with a range of 0-10 pounds was used for testing more mature fruits. Pressure readings were expressed in pounds as the average of the 10 determinations. After the pressure-test determination the peaches were pitted and then macerated in a Waring blender for one minute. The titratable acidity was determined by neutralizing 10 g. lots of the macerate suspended in 200 ml. of distilled water to pH 8.0 with 0.1 N NaOH. Total solids was determined by drying 10-20 g. of the macerate at 70°C. for 16 hours under a vacuum of 27 inches.

Paper chromatography of the organic acids. The organic acids in the canned peaches were identified by the paper chromatographic method developed by Lugg and Overell *(14)* and used also by Hulme and Swain *(7)*. The canned peach samples were macerated and filtered through Whatman No. 1 filter paper in a Buchner funnel under suction. A 0.01 ml. aliquot of the filtrate was directly applied to Whatman No. 1 filter paper.

A descending technique using a *n*-butanol–formic acid–water (40:10:50 v/v) solvent system at 70°F. (20°C.) for 16 to 20 hours was employed. The solvent mixture was allowed 8 hours' equilibration before use. Only the top layer of the solvent mixture was used. The chromatograms were developed with 0.04% bromophenol blue in 95% ethanol, which had been adjusted to pH 6.8 with 0.1 N NaOH. The acids appear as yellow spots on a blue background.

Isolation of quinic acid. Quinic acid was isolated from four No. 2½ cans of Fay Elberta peaches. Each can contained 19.5 oz. of peaches and 10.5 oz. of 40° Brix sucrose syrup. The sample was macerated in a Waring blender, frozen, and lyophilized in a Stokes freeze-drying unit under high vacuum. The dried material was extracted with 3 liters of 95% ethanol on a steam bath for 30 minutes. The mixture was filtered and the residue extracted with additional alcohol. Then 300 ml. of water was added to the filtrate. The filtrate and the washings were combined and evaporated to about 1,000 ml. on a steam bath. Deactivated charcoal, prepared according to Partridge's method *(22)*, was added. The mixture was heated to boiling and filtered. The filtrate was made up to a volume of 900 ml. with distilled water.

Dowex 1, an anion-exchange resin, with 8% cross linkage and 100-200 mesh particle size, was prepared in the hydroxide form and packed into a column 50 cm. long and with an inside diameter of 2.2 cm. A 650 ml. aliquot of the extract was run over the column at a rate of approximately 100 ml. per hour. The column was washed with CO_2-free distilled water until a negative test for sugars was given with Benedict's solution. Then 800 ml. of 0.05 N HCl, followed by 100 ml. of 0.1 N HCl solution, was run over the column at a rate of approximately 100 ml. per hour. The eluate was collected

as fractions with volumes varying in size from 20 ml. to 75 ml., the pH change being used as a guide to the composition of the fractions eluted from the column *(8)*. Quinic, malic, and citric acid, in that order, were eluted from the column with fairly good separation.

All the quinic acid appeared in the fractions eluted with 0.05 N HCl. The fractions containing quinic acids were combined and concentrated under vacuum, and the acid was allowed to crystallize from ethanol and water. Recrystallization from ethanol and ethyl ether yielded 300 mg. of white prismatic crystals of quinic acid.

Quantitative determination of malic and citric acids. For the estimation of malic and citric acids, a silicic acid column chromatographic procedure of Bulen, Varner, and Burrell *(4)*, which is a modification of the techniques of Marvel and Rands *(17)* and Isherwood *(10)*, was employed.

For this estimation 2 cans of peaches were macerated in a Waring blender for 1 minute. The mixture was filtered. A 10-ml. aliquot of the filtrate was acidified to 0.5 N with respect to H_2SO_4. Two g. of silicic acid were weighed into a 30 ml. beaker and a 1 ml. aliquot of the acidified filtrate was added. The juice and silicic acid were thoroughly mixed. The resulting mixture was transferred to the top of the column as described by Bulen *et al. (4)*. Solvent mixtures of 25% and 35% *n*-butanol in chloroform (v/v) were run consecutively, and 5 ml. fractions were collected and titrated against 0.01 N NaOH to a phenol red end point. The amount of each acid is expressed as milliequivalents per 100 g. of raw fruit adjusted to a total solids content of 13.6%. Duplicates were run on each sample.

Determination of quinic acid. The procedure used to estimate citric and malic acids was found to be impractical for quinic acid. However, a guide to its concentration was obtained by the following method: Various water dilutions of the peach filtrates were prepared. These were applied as spots in 0.01 ml. amounts to the top of Whatman No. 1 filter paper and the chromatograms developed as described earlier. The spots were detected with bromophenol blue and the papers were examined under a standard light. The dilution at which the acid spot on the paper became indiscernible was noted for each sample. The procedure was repeated three times and the mean dilution value obtained.

Standard quinic acid solutions were treated identically and the extinction concentration of quinic acid was determined. This was found to occur at a concentration of 3 gamma per 0.01 ml. From the dilutions required, the concentration of quinic acid could be estimated. It should be remembered that these values only give the order of the concentration and are not a precise determination.

RESULTS AND DISCUSSION

Identification of organic acids in peaches. The major organic acids in canned Fay Elberta freestone peaches were investigated qualitatively by the paper chromatographic method described by Lugg and Overell *(14)* and Hulme and Swain *(7)*. Three distinct yellow spots with Rf values of 0.20, 0.38, and 0.50 were identified as quinic, citric, and malic acid, respectively, by comparison with authentic known samples. In addition to these major acids, a faint spot with a Rf value of 0.08 was found, which was thought to be either galacturonic or mucic acid. The presence of mucic acid in raw peaches has also been reported by Anet and Reynolds *(2)*. On paper chromatograms of the first few fractions containing quinic acid, which were eluted from Dowex 1 anion exchange resin as described previously, three faint spots appeared at Rf values of 0.04, 0.12, and 0.75 besides the quinic at Rf 0.20. The first is unknown, the second may be aspartic, and the third is probably lactic acid which Hulme *(9)* found to result from the interaction of the basic resins and sugars.

The following evidence indicates that acetic acid or acetate ion may also be present: (a) by silicic acid column chromatography, a peak corresponding to acetic acid *(4)* comes down when a solvent made of 5% *n*-butanol

in chloroform (v/v) was used as eluting agent; (b) no acetic acid was found when the eluate from this peak was tried by paper chromatographic methods, indicating that the compound is volatile; and (c) recent work by McCready and McComb *(15)* had indicated the presence of acetyl groups up to 3% in the pectins of both ripe and unripe Fay Elberta peaches. It is quite possible that acetic acid was derived from the pectin in the peaches. This point needs further investigation.

Other organic acids, such as ascorbic, chlorogenic *(11)*, neo-chlorogenic *(5)*, salicylic *(25)*, and tartaric *(21)*, have also been reported as occurring in peaches. Although Nuccorini and Cerri *(21)* reported the presence of tartaric acid in peaches in amounts greater than citric acid, the presence of tartaric acid was not indicated during the course of this work. Possibly this might be attributed to the non-specificity of their methods. No evidence of chlorogenic, neo-chlorogenic, or salicylic acid was detected on the paper chromatogram. These acids may be present in a concentration too low to be detected by present methods of investigation, or they may have been changed into other constituents during processing.

The presence of quinic, citric, and malic acid as major acids in Halford and Peak clingstone peaches was similarly demonstrated.

Identification and estimation of quinic acid. The acid isolated from Fay Elberta freestone peaches by the method described above was shown to be quinic by the following evidence: (a) Its Rf value agreed with that of an authentic sample of quinic acid (Rf = 0.20); (b) only one spot was obtained when a mixture of the isolated and authentic acid was chromatographed; and (c) the melting point of the isolated acid agreed with that of an authentic sample. The isolated acid when crystallized from ethanol and ether melted at 169°-172°C., while an authentic sample when recrystallized from the same system melted at 169°-171°C. The melting point did not drop when the two samples were mixed. These data support the identity of quinic acid, which was reported to have a melting point of 172°C. according to Heilbron *(6)* and 168°C. by Anet and Reynolds *(1)*, although Hulme *(8)* reported 160°C. for l-quinic acid derived from apples.

Quinic acid was found in canned samples of Halford, Peak, Fortuna, and Gaume clingstone peaches and in July Elberta and Fay Elberta freestones. Paper chromatograms of water extract of frozen Elberta peaches also show the presence of quinic acid, indicating that it is present in the raw fruit and not a decomposition product due to processing. On the basis of this work in which quinic acid was found in all 6 varieties, and from the results of Anet and Reynolds *(1)*, it may be concluded that this acid is widely distributed among the various peach varieties.

The change of quinic acid with maturation is shown in Figures 2, 3, and 5. The concentration of quinic acid is approximately the same for each variety at the hard green stage and then decreases during maturation. This acid represents 15% of the total acid (sum of malate, citrate, and quinate in milliequivalents) for Halfords and Fay Elbertas and 5% of that for the Peak variety at harvest maturity. These values are less than those reported by Anet and Reynolds *(1)*, who obtained a quinic acid concentration varying from 9 to 24% of the total acids for ripe fruit of other varieties. Such differences might be attributed to variety, maturity,

climatic condition, or the method used for determination, or possibly to a combination of all these factors.

Changes in organic acids of clingstone peaches during maturation. The quantitative relationship between the major organic acids and total titratable acids in canned Halford and Peak varieties, when correlated with pressure test of the fresh fruit as an index of maturity, is shown in Figures 1, 2, and 3. Since the moisture content of the fruit varied somewhat at various stages of maturity, all values were adjusted to a total solids of 13.6%.

Broadly speaking, the pattern of changes in total titratable acidity and in malic and citric acid concentrations is the same for both varieties for each year. They increase to a maximum concentration during fruit growth and decrease at harvest maturity. This general trend has also been reported by other workers, *(12, 13, 19, 20, 23, 24)*. Peynaud *(23)* did observe, however, that the total titratable acidity and malic acid of the J. H. Hale variety gradually increased during maturation, while the citric acid increased up to 2 weeks before harvest and then levelled off. With other varieties he noticed a rise in the acids to a peak at the pre-ripe stage and then a dropping off, observing a greater drop in the citric than in the malic acid concentration.

Results presented in Figure 1 indicate that citric acid and total titratable acidity decreased rapidly during maturation when the pressure test

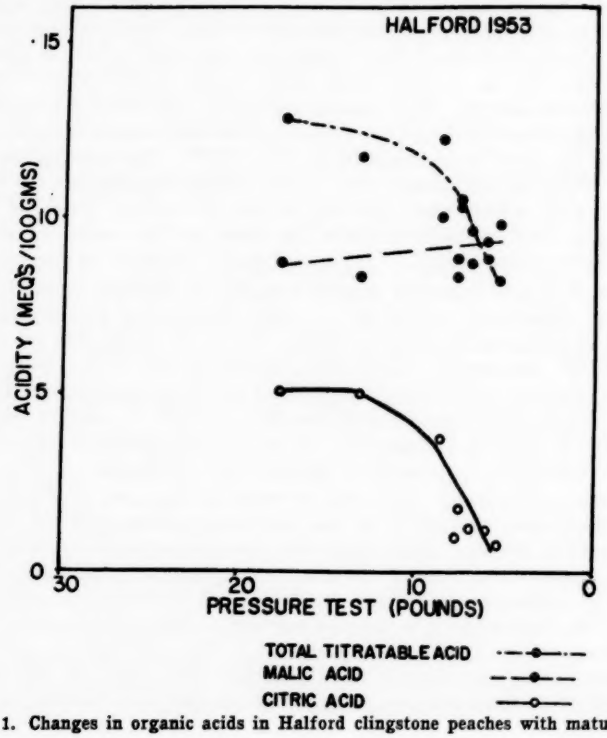

Figure 1. Changes in organic acids in Halford clingstone peaches with maturation.

dropped from 18 lbs. to 6 lbs. Malic acid showed no particular trend. In Figure 2, data on Halford peaches packed during 1954 are presented. A greater number of samples covering a wider maturity range than that of the 1953 season was used. Hence, we see a better pattern of acid changes during maturation. The malic acid content increased with maturation to a pressure test of 11 pounds and then gradually decreased to a lower value at the harvest date. If the changes in acids as shown in Figures 1 and 2 were compared over the same pressure range of 18 down to 6 pounds, the trends for citric acid and total acids would show a similar pattern for both years.

The pattern of changes in acids during maturation of the Peak variety is shown in Figure 3. The overall behavior is somewhat similar to that in the Halford. Total acidity at canning maturity is, however, higher than in the Halford, primarily because of its higher malic acid content. In contrast to the Halford, the Peak variety does not reach its maximum acidity until it is much more mature, as determined by pressure test. In Figure 3, the points numbered 1, 2, and 3 represent the total titratable acidity, malic, and citric acid concentrations, respectively, of a sample of Peaks picked 5 days after the harvest date and stored for 24 hours at room temperature

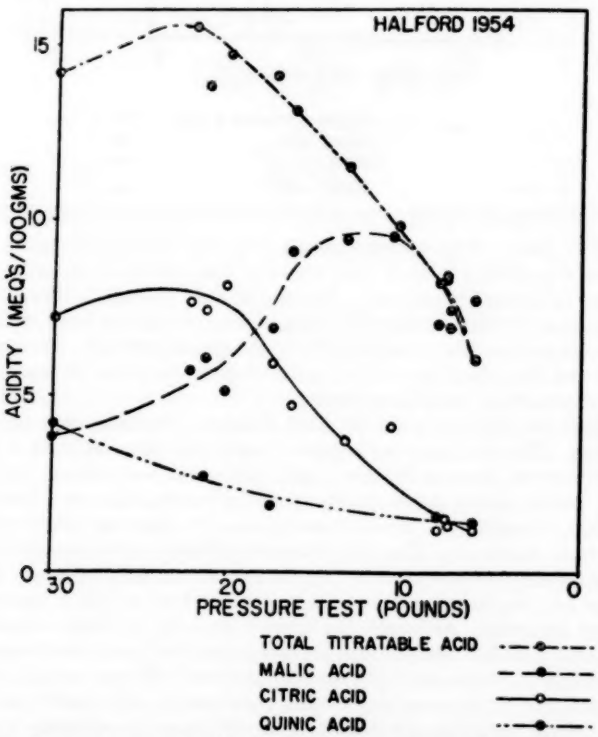

Figure 2. Changes in organic acids in Halford clingstone peaches with maturation.

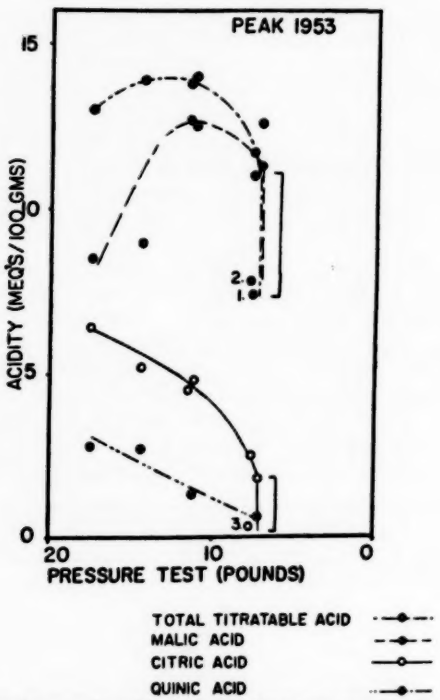

Figure 3. Changes in organic acids in Peak clingstone peaches during maturation.

in the pilot plant. The concentrations of malic and citric acids in this sample are significantly lower than those of the previous unstored samples. This large difference is probably due to the high storage temperature and the additional length of time the fruit remained on the tree. It is interesting to point out here that the Peak clingstone variety, in contrast to freestone peaches, does not soften below 7 pounds pressure test even five days after reaching canning maturity.

Changes in organic acids in Fay Elberta freestone peaches during maturation. The changes in organic acids during maturation of Fay Elberta freestone peaches for 1953 and 1954 seasons are shown in Figures 4 and 5. Here again, total acidity and the concentrations of malic and citric acids increased to a maximum prior to ripening, then decreased until harvest maturity. The Fay Elberta differed from the Halford and Peak clingstone peaches in that (a) its maximum acid concentration was lower and (b) its citric acid was not metabolized to so low a concentration at harvest maturity. Although the overall pattern for acid metabolism in Fay Elberta peaches was similar for both the 1953 and 1954 seasons, the maximum values reported between the 2 years differed considerably. A more rapid rise to the maximum value occurred in 1954 and a slower rise in 1953. This could be attributed to a difference in climatic conditions between the 2 years.

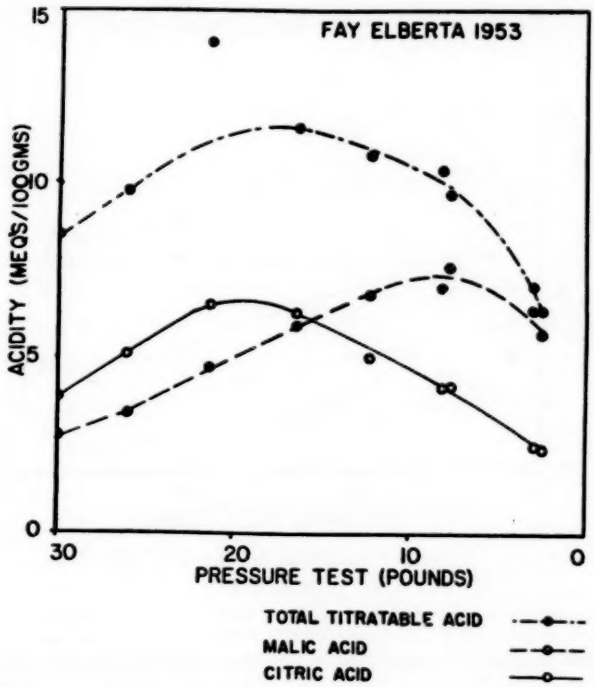

Figure 4. Changes in organic acids in Fay Elberta freestone peaches during maturation.

Ratio of malic to citric acid in peaches. The variation in the ratio of malic to citric acid during the course of maturation of both clingstone and freestone peaches is shown in Figure 6. Even though there appears to be some difference in the acid changes of the different varieties over the 2 years, the ratio of malic to citric acid (based on milliequivalents per 100 g.) is remarkably consistent. All the varieties exhibit very similar trends until they reach a pressure test of approximately 14 pounds. At this stage the values for the clingstones increase rapidly, while the rate of increase for the freestones remains constant. The point at which the curves begin to differ occurs about 17 days before harvest for the clingstones and about 10 days before harvest for the freestones. At canning maturity and pressure test of about 8 pounds, the ratio for the clingstones increased to a value above 6.0, while that of the freestone variety at pressure test of 2 to 3 pounds had a value of only 2.0. This is an important difference between clingstone and freestone peaches at canning maturity, which is due to a lower citric acid content in the clingstones. It remains to be investigated whether the difference in flavor between clingstone and freestone is due only to their difference in acid content at canning maturity or to differences in other flavor constituents.

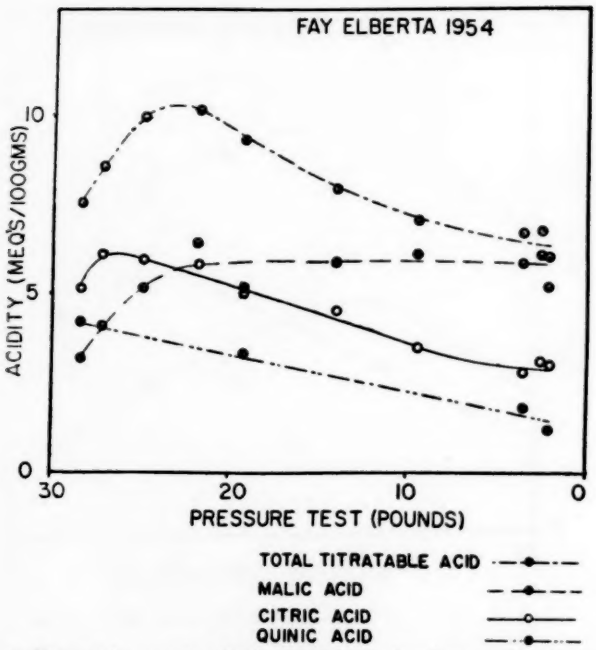

Figure 5. Changes in organic acids in Fay Elberta freestone peaches during maturation.

Peynaud *(24)* reported a malic to citric acid ratio of 1.4 for mature Early Elberta peaches and 2.8 for mature July Elbertas. This is in good agreement with the values obtained in this study for the presumably similar Fay Elberta variety. Peynaud *(23)* has also reported ratios varying from 0.78 to 4.7 for peaches grown in France, with citric acid predominating over malic in only two of the varieties studied.

The sum of malic, citric, and quinic acids present in peaches was found always to be greater than the total titratable acid. This was also observed by Peynaud *(23)* who found that the sum of the acids was greater than the titratable acid depending on the variety and maturity. The method used in this work actually determines the sum of the free acid and the anions of each individual acid. The titratable acidity, which measures only the total free acids, thus shows a lower value.

The work of Leonard, Luh, and Hinreiner *(12)* and the results described above show that the decrease in organic acids with maturation is related to the quality of the canned peaches. It is possible that some of the flavor constituents in peaches, which are produced when the fruit ripens, may be associated with metabolism of organic acids.

SUMMARY

Halford and Peak clingstones and Fay Elberta freestone peaches were investigated in two consecutive years for their change in organic acids

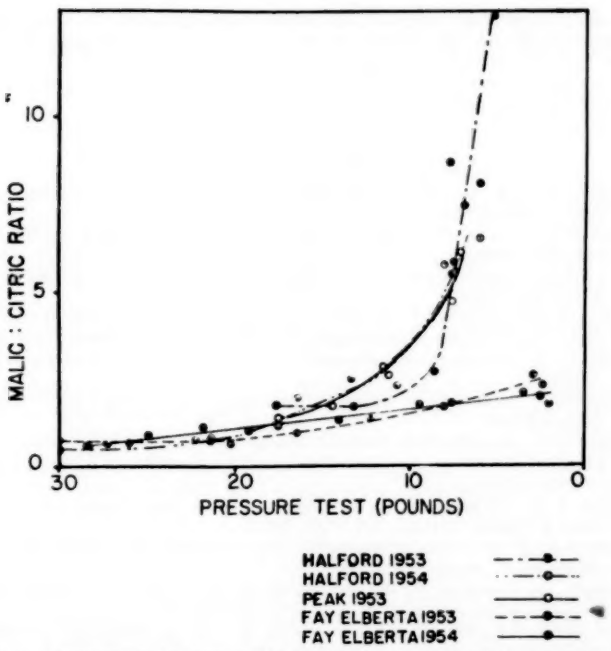

Figure 6. Relationship between malic to citric acid ratio and pressure test of the fresh peaches.

during growth and maturation on the tree. The peaches were harvested at different stages of maturity as judged by pressure test, canned in distilled water, and then analyzed for citric, malic, and quinic acids by the silicic acid column or paper chromatographic method. Citric, malic, and quinic acids were found to be the major acids present in both clingstone and freestone peaches, although the possible presence of acetic, aspartic, and mucic or galacturonic acid was also indicated. The total titratable acidity and the concentrations of malic and citric acids increased to a maximum during fruit growth and decreased during maturation.

It is characteristic of clingstones that citric acid decreases more rapidly than malic acid during maturation. The ratio of malic to citric acid of clingstones at canning maturity and pressure of 8 lbs. was greater than 6.0, while that of freestone peaches at pressure test of 2 to 3 lbs. was only 2.0. The importance of such changes to the quality of canned peaches is discussed.

A method for the separation of quinic acid from canned peaches by alcohol extraction, fractionation on Dowex 1 anion exchange resin column, and crystallization from ethanol-ether mixture is described.

Acknowledgment

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FREEZE-DRIED MEAT. II. THE MECHANISM OF OXIDATIVE DETERIORATION OF FREEZE-DRIED BEEF[a]

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Dehydrated meats of good organoleptic quality and functional properties can be produced in the form of steaks, chops, roasts, and stew meat by the method of freeze-drying *(3, 13)*. Since the primary value of freeze-dried meat, especially for the military forces, lies in its storage stability at elevated temperatures, studies of its mechanism of deterioration become important.

The mechanisms of deterioration of freeze-dried meat may be conveniently divided into those of oxidative and those of non-oxidative deterioration. Since the oxidative deterioration of such dehydrated foods as meat, milk, and eggs can be prevented by commercial gas packing the mechanisms of this deterioration have not been completely investigated *(2, 9, 11)*.

Except for the work of the British, which has been recently reviewed by Sharp *(11)*, the information available on oxidative deterioration of dehydrated meats is generally meager. In precooked air and vacuum dehydrated pork, the oxidation of unsaturated fatty acids is known to play a dominant role. It has been recognized that the oxidation of the non-lipid fraction especially in dehydrated beef is also an important mode of oxidative deterioration. This mechanism of the non-lipid oxidative deterioration of dehydrated meat has not been investigated. Principally because of oxidative pigment changes, the oxidative deterioration of freeze-dried raw meat assumes greater significance than the oxidative deterioration of dehydrated meat which was produced at high temperatures.

The rate of oxygen absorption, the effect of oxygen on the flavor, and changes in peroxide value of freeze-dried raw beef have been measured by the British workers *(11)*. Available oxygen had a pronounced detrimental effect on flavor of freeze-dried beef held in storage at 37°C. and 15°C. The loss of the bright pink color of freeze-dried beef was noted. It was found that the large oxygen absorption and the corresponding deterioration could be prevented by tight packing of the minced freeze-dried meat in cans.

This paper reports research directed toward establishing the mechanism of the oxidative deterioration of freeze-dried beef. It was found that the protein portion of freeze-dried beef was more oxygen labile than was heretofore expected. Oxidation of the non-ether-extractable lipids appears to

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[a] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 587 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

account for half of the oxygen absorption. The oxidation of ether soluble lipids plays a minor role. No evidence was found for oxidative browning reactions. Oxidative discoloration of hematin pigments is an important problem.

EXPERIMENTAL

Freeze-dried meat. The *biceps femoris* muscle of beef animals was used throughout the course of this study. The freeze-drying of meat has been previously described *(13)*. Freeze-dried meats were equilibrated to desired vapor pressures over solutions of sulfuric acid in controlled temperature chambers.

Reflectance and absorption spectra. Reflectance spectra were measured with a rapid scanning spectrophotometer.[b] For absorption spectra, aqueous extracts of freeze-dried meat were prepared from meat which had been ground in a Wiley Mill to pass 10 mesh and extracted twice with 10-ml. portions of diethyl ether per ½ g. of beef. One-half g. of beef was ground in powdered glass and dissolved in 10 ml. of distilled water and after centrifugation the spectra in the region of 460-480 mμ were measured. For measurement of the spectra in the region 380-450 mμ the extract was diluted 5 times.

Oxygen absorption. Oxygen absorption at 38°C. was measured using a Warburg respirometer. In the majority of these studies the main compartment of the 100 ml. reaction flask contained 10 g. of freeze-dried beef, and for CO_2 absorption the side compartment contained 1 ml. of concentrated potassium hydroxide equilibrated to the desired vapor pressure. For a measurement of carbon dioxide evolution, the potassium hydroxide solution was added at the end of the reaction period. When the reaction was measured for extended periods of time, the aqueous manometer fluid was replaced with butyl phthalate which has a negligible vapor pressure. In all studies the oxygen absorption was followed for a suitable length of time, usually 35-150 hr., so that reaction rates could be accurately determined from the plotted data.

Oxidation of lipids. At predetermined levels of oxygen absorption ground freeze-dried beef was removed from the Warburg flasks and stored under nitrogen at −20°C. prior to analysis. For the determination of monocarbonyl compounds ½ to 1 g. of meat was extracted with benzene in a small Bailey-Walker type of extractor. The extracted carbonyl compounds were allowed to immediately react in the boiling benzene solution with the 2,4-dinitrophenyl hydrazine. The analysis for monocarbonyl compounds was carried out according to the method of Pool and Klose *(10)*. The remaining 9 g. of beef was extracted for 8 hours in a Soxhlet apparatus with peroxide free petroleum ether. The petroleum ether was removed by distillation in vacuum with a stream of nitrogen. The iodine number was determined by the Wijs method. Peroxide values were determined iodometrically. All reactions were carried out under nitrogen.

Lipid extractions. Besides the extraction of ether soluble lipids by 12 hr. Soxhlet extractions using diethyl ether, more rigorous lipid extractions were used. In general, these 4 methods are sufficiently rigorous to assure some hydrolysis of the compound or conjugate lipids, so that complete extraction of lipid material is facilitated. These methods are as follows:

1. This method is a modification of the methods for fish meal fat *(8)*. Freeze-dried beef, ground to pass 10 mesh, was extracted with acetone by the continuous batch extraction for 12 hours using the Soxhlet apparatus.
2. This method is essentially the method for the extraction of lipoids from cereal foods *(8)*. Twenty grams of ground freeze-dried beef were extracted with 100 ml. of 70% aqueous ethanol at 75°C. for 15 min. Then, 100 ml. of absolute ethanol were added and the mixture shaken 2 min. After cooling, 200 ml. of ethyl ether were added and this mixture was shaken 5 min. The freeze-dried beef was filtered in a Buchner funnel and dried under vacuum.
3. This method is essentially the method for the extraction of lipoids from dried eggs *(8)*. Twenty grams of ground freeze-dried beef were extracted for 12 hrs. at room temperature with 450 ml. of 95% ethanol and 450 ml. of chloroform. The freeze-dried beef was filtered and the solvents were removed under vacuum.

[b] American Optical Company.

4. This combination method consisted of a 21 hr. ether extraction followed by further extraction with 95% ethanol and chloroform for 1 hr. at room temperature.

Chemical additions to freeze-dried beef. The dry-mixed freeze-dried beef and chemical additives were rehydrated in 4 ml./g. distilled water, wet-mixed, immediately frozen in pans, and freeze-dried. Following freeze-drying the freeze-dried beef with chemical additives was equilibrated to the desired vapor pressure.

Preparation of protein fractions. This extraction was designed to remove all lipids and all water soluble components from freeze-dried meats so that only the protein fraction remained. Ground, diethyl ether-extracted, freeze-dried beef, lamb, and chicken were extracted twice with 50% aqueous ethanol at 25°C. for 10 min. each. The meat swelled and rehydrated somewhat but was readily separated from the solvent by centrifugation at 1000 × gravity. Under these fractionation conditions all water soluble solutes should be removed. The protein is not soluble and because of the dehydration effects of ethanol at these temperatures the protein is probably denatured during this extraction. Following the aqueous ethanol extraction the meat protein was treated with absolute ethanol at 70°C. for 5 min. and finally the meat proteins were extracted with diethyl ether and dried in vacuum.

RESULTS AND DISCUSSION

The occurrence of oxidative deterioration in freeze-dried beef. In exploratory studies the rapid browning of freeze-dried beef stored in excess air at 38°C. was noted. The initial pink-red color of freeze-dried beef changed to red-brown after 10 hr. The beef became a definite brown after 3 days and after 20 days the intensity of the brown color decreased as the color changed toward a yellow-brown.

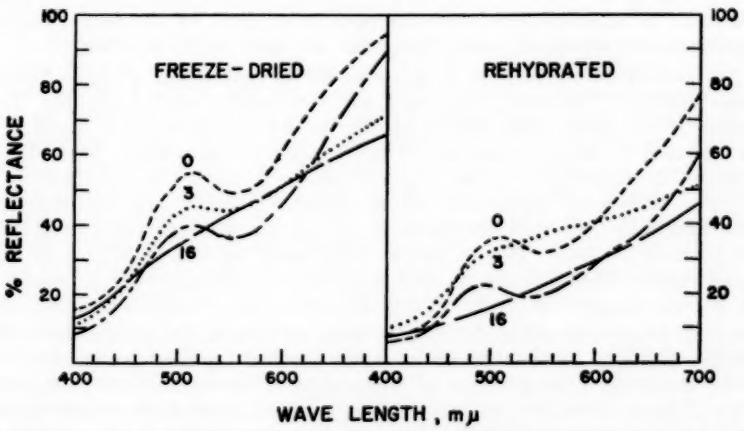

Figure 1. The reflectance spectra of freeze-dried beef after storage in air at 38°C. for 0, 3, and 16 days. The spectra of freeze-dried beef is compared with that of dry beef oxyhemoglobin; the spectra of rehydrated freeze-dried beef is compared with that of the fresh beef control.

Figure 1 shows the change in the reflectance spectra of freeze-dried beef as oxidative deterioration progresses. There was a loss of some of the spectral characteristics of myoglobin and hemoglobin after 3 days. The reflectance maximum at 510 mμ decreased and the minimum at 560 mμ

became less pronounced. After 16 days storage the spectral characteristics were entirely lost and the spectral reflectance is typical of a browned food product. The reflectance spectra after rehydration show more pronounced changes in the beef which had been stored for 3 days in air. The oxymyoglobin characteristics were absent and the meat was a brown metmyoglobin color. Rehydration of the beef stored 16 days gave a darker brown color and its spectral reflectance showed some loss in the minimum at 410 mμ which is a well known characteristic of all hematin compounds. Corresponding samples held in nitrogen or carbon dioxide developed a brown color more slowly. These observations on browning in different gas atmospheres clearly indicate that oxidative reactions are involved in the storage deterioration of freeze-dried beef.

Exploratory studies of the quantity of oxygen absorbed during the deterioration of freeze-dried beef showed oxygen consumption of 2.5 ml./g./month at 38°C. for meat equilibrated to a water vapor pressure of 18.4 mm.

Oxidation of myoglobin and hemoglobin. During the freeze-drying process the muscle oxymyoglobin and any residual oxyhemoglobin are exposed to total pressures of 0.2 mm. Hg. or less, and hence, are largely deoxygenated to myoglobin and hemoglobin *(7)*. The relative amounts of myoglobin and hemoglobin were not measured. Myoglobin accounts for approximately 90% of the total hematin compounds in fresh meat *(16)*.

In freeze-dried beef the myoglobin and hemoglobin apparently remain deoxygenated even in atmospheres of pure oxygen because there is no observable color difference between freeze-dried beef under high vacuum or under an atmosphere of pure nitrogen or oxygen. In the deoxygenated condition dry myoglobin and hemoglobin are very labile to oxidation.

Freeze-dried beef stored in gases at 38°C. underwent a rapid brown discoloration in the order: oxygen $\geqq$ air $>$ nitrogen $=$ carbon dioxide. Because of the rapid development of this brown discoloration it could not be ascribed to the end products of active-aldehyde browning. This observation, the studies of reflectance spectra shown in Figure 1, and the known oxygen lability of myoglobin and hemoglobin suggest that oxidation to metmyoglobin and methemoglobin is one of the first reactions occurring in freeze-dried beef. This oxidation could occur by either or both of two mechanisms. First, the dry myoglobin and hemoglobin could lose an electron to gaseous oxygen. Second, the dry myoglobin and hemoglobin could lose an electron to some other electron acceptor present in the freeze-dried beef itself. The first mechanism undoubtedly predominates when freeze-dried meat is stored in the presence of free oxygen. The rapid brown discoloration of freeze-dried beef stored in nitrogen atmospheres from which oxygen was excluded suggests that the second oxidative mechanism is also operative. This second mechanism is practically the most important because commercial storage of freeze-dried beef will require exclusion of oxygen for maximum product stability.

Further evidence that the initial brown discoloration of freeze-dried meat is metmyoglobin was found in experiments on the reversibility of the reaction and the formation of myoglobin derivatives which prevent this oxidative reaction *(1)*. When ascorbic acid was incorporated in the water for rehydration of freeze-dried beef which had undergone the initial brown

discoloration, metmyoglobin and methemoglobin were reduced to myoglobin and hemoglobin. In the presence of oxygen the myoglobin and hemoglobin were oxygenated to give rehydrated beef with the color of fresh meat. The conversion of the hematin pigments in freeze-dried beef to carboxymyoglobin and carboxyhemoglobin was easily accomplished by exposing the freeze-dried beef to carbon monoxide gas. Freeze-dried beef treated with carbon monoxide had a very stable bright pink color when stored at 38°C. or 55°C. under an atmosphere of pure nitrogen. Only after 2 months at 55°C. did the brown color of the browning reaction develop. Freeze-dried beef with nitrosomyoglobin was produced by freeze-drying ground beef patties treated with nitrite, ascorbic acid, and copper ions. Freeze-dried beef with nitrosomyoglobin has a stable cured-meat pink color when stored at 38°C. in an atmosphere of pure nitrogen.

When freshly prepared freeze-dried beef was rehydrated in the presence of air, most of the rehydrated myoglobin and hemoglobin became oxygenated and a color similar to fresh meat was obtained. However, some of the rehydrated myoglobin and hemoglobin was oxidized producing the brown discoloration characteristic of metmyoglobin and methemoglobin. The relative amounts of oxygenation and oxidation obtained are mainly a function of the oxygen partial pressure. At very low oxygen tension, rehydration produces beef having the brown color of metmyoglobin. Rehydration in water saturated with oxygen gives the natural bright red oxymyoglobin color. Consequently, it is good practice to rehydrate freeze-dried beef in well oxygenated water so that beef of natural color may be obtained.

In contrast to freshly prepared freeze-dried beef, that subjected to any adverse condition not rigorous enough for the production of the initial brown discoloration of hematin compound oxidation became increasingly labile to oxidation during the rehydration process. The extreme sensitivity of the oxygenation mechanism of myoglobin and hemoglobin to small changes in the globin moiety suggests that slight changes in the globin during storage eliminate the possibility of oxygenation. Therefore, the slightly altered dry myoglobin and hemoglobin can only undergo oxidation after rehydration. The sensitivity of freeze-dried beef stored under a wide range of temperature and moisture conditions to metmyoglobin and methemoglobin formation is shown in spectral absorption studies.

The absorption spectra of aqueous extracts of freeze-dried beef which had been stored for four months under an atmosphere of nitrogen show in Figure 2 the presence of large amounts of metmyoglobin and methemoglobin. The metmyoglobin and methemoglobin maxima at 500 mμ and 630 mμ give quantitative information on the amount of oxidation during storage and rehydration *(7)*. The sample stored at –20°C. appeared visually to retain most of its initial pink-red color before rehydration. Samples containing in-package desiccant or 3% water show increasing losses of the characteristics of oxymyoglobin, i.e. a decrease in the maxima at 540 mμ and 580 mμ, and relatively high concentrations of metmyoglobin. The total decrease in spectral absorption and loss of spectral characteristics as a function of water content is caused by insolubility as a result of the increased browning reaction. Thus, the aqueous extract of the sample

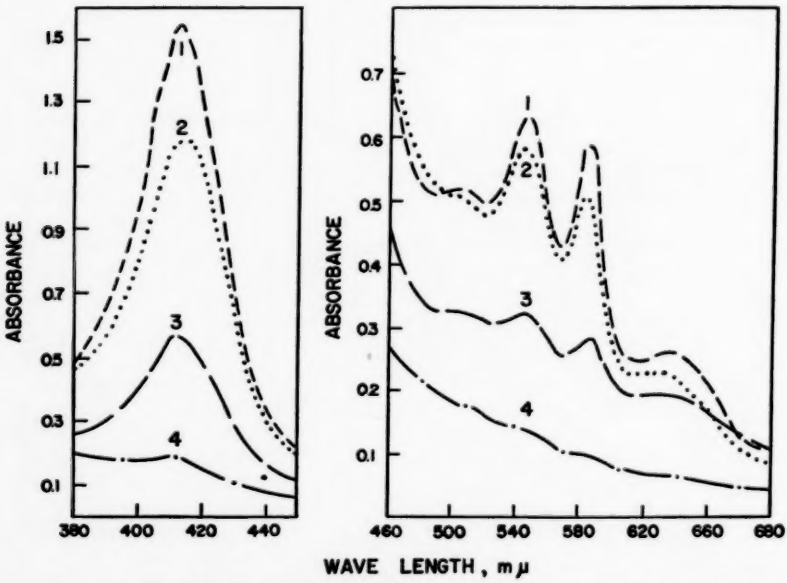

Figure 2. The absorption spectra of aqueous extracts of freeze-dried beef stored for four months in a nitrogen atmosphere.

1. **−18°C., 3% water.**
2. **38°C., in-package desiccant.**
3. **38°C., 3% water.**
4. **38°C., 8% water.**

containing 8% water showed very slight maxima and this is indicative of almost complete loss of soluble oxygenated and oxidized hematin pigments.

The effects of freeze-drying, storage, and subsequent rehydration of the hematin pigments is outlined for myoglobin in Figure 3.

The importance of unsaturated lipid oxidation. Unsaturated lipids are the only well-known oxygen-labile reactants present in beef which could

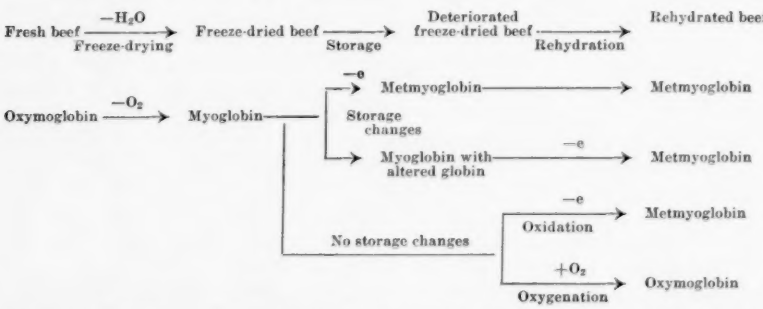

Figure 3. Changes in myoglobin during freeze-drying, storage, and rehydration of beef.

readily account for the rapid and large oxygen absorption noted. Therefore, this reaction immediately became suspect. Unsaturated fat oxidation generally increases with decreasing water content *(11)*, and the probability of surface catalysis *(6)* and hematin catalysis *(12)* must be considered. The known end products of fat oxidation, mainly carbonyl compounds, are reactants in the browning reaction and could account for the relation between oxygen absorption and the general deterioration of freeze-dried beef.

Indirect evidence in favor of the fat oxidation hypothesis came from the inhibition of oxygen absorption of freeze-dried beef by fat antioxidants (Table 1).

TABLE 1

The effect of adding polyphenolic antioxidants on the rate of oxidation of freeze-dried beef

Antioxidant	Concentration %	Rate of oxygen absorption, % of control
Nordihydroguaiaretic acid	0.05	52
Nordihydroguaiaretic acid	0.5	60
Propyl gallate	0.05	82
Propyl gallate	0.5	73

The powerful antioxidants nordihydroguaiaretic acid and propyl gallate were inhibitory at the low concentrations of 0.05% but higher concentrations did not increase this inhibition. Based on maximum inhibition produced, and the fact that these antioxidants function by donating hydrogen to free-radicals this study suggests that about half the oxygen absorption could be ascribed to a free-radical oxidation of some lipid component.

To obtain a more direct evaluation of the importance of unsaturated fat oxidation in the storage deterioration of freeze-dried beef, the oxygen absorption of the freeze-dried beef was correlated with the concentration of peroxides and monocarbonyl compounds and the iodine value of the ether extracted fat. The resulting analytical values are tabulated as a function of time in Table 2. For a comparison with direct oxygen absorption, the peroxide value, carbonyl content, and the decrease in the iodine value are also expressed in terms of equivalent oxygen. In this comparison it was assumed that a decrease in iodine value is indicative of oxidative

TABLE 2

Comparison of oxygen absorption with increase in peroxide and carbonyl concentrations and decrease in iodine number

Time, hours	O_2 uptake ml./g. meat	Peroxide value, Milliequivalents / 1000 g. fat	Monocarbonyls moles/g. meat	Iodine No., I_2 g./100 g. fat	O_2 uptake mg./g. meat	Peroxide oxygen	Carbonyl oxygen	Decrease in I_2 No.
0	0.	3.0	11.2×10^{-7}	59.8	0	0	0	0
30	0.46	....	18.2×10^{-7}	.....	0.66	.....	0.0112	.....
89	0.88	4.1	20.0×10^{-7}	59.5	1.26	0.0045	0.0157	0.048
187	1.38	4.8	21.6×10^{-7}	59.4	1.97	0.0074	0.0166	0.064
319	1.81	4.8	21.8×10^{-7}	58.0	2.59	0.0074	0.0170	0.288
463	2.26	5.8	21.6×10^{-7}	58.5	3.22	0.0115	0.0166	0.208

scission of the unsaturated fatty acids at the double bond. The direct products of unsaturated fat oxidation, i.e. fat peroxides and carbonyl compounds, can only account for about 1% of the oxygen absorption of the freeze-dried beef. The low levels of peroxide can be ascribed to either of two causes. The amount of fat oxidation may be small or peroxides may be unusually labile in freeze-dried beef. Likewise, a small amount of fat oxidation or the further reaction of carbonyl compounds in the browning reaction could account for the low levels of carbonyl compounds found. At most, the decrease in iodine value noted could account for 11% of the oxygen absorption. Clearly, the total oxygen absorption cannot be accounted for in terms of oxidation of ether soluble unsaturated fat.

Stronger evidence against oxidation of the unsaturated fat in the ether extractable fraction was obtained from studies of the oxygen absorption of extracted freeze-dried beef. The results show (Table 3) that the fat

TABLE 3

The oxygen absorption of freeze-dried beef after 35 hours at 100°F.

Freeze-dried beef sample	Oxygen absorption ml./g. fat-free beef
Ether extracted	0.42
Ether extracted + natural fat	0.43
Ether extracted + tricaproin	0.46

content had no significant influence on the rate of oxygen absorption. On the basis of this result it may be concluded that diethyl ether extractable lipids are not responsible for the large oxygen absorption of freeze-dried beef.

The non-ether soluble phospholipids, lipoproteins, etc. are known to be highly unsaturated and could be the reactants in the oxygen consuming deterioration of freeze-dried beef. To test this possibility samples of rigorously extracted freeze-dried beef were prepared as previously described and their oxygen absorption was compared to unextracted and ether extracted beef. The results in Table 4 show that rigorous extraction of all

TABLE 4

The rate of oxygen absorption of extracted freeze-dried beef

Extraction	Rate cu. mm. O_2/g./hr.
None	10.3
12 hr. diethyl ether	13.4
12 hr. acetone	7.7
15 min. 168-176°F. ethanol	7.0
12 hr. diethyl ether + 1 hr. ethanol + chloroform	7.0
12 hr. ethanol + chloroform	5.7

lipids did not eliminate the oxygen absorption. Because of similarities of particle size distribution and structure the samples with total lipid extracted should be compared with the ether extracted beef. It is significant that the rigorously extracted samples had oxygen absorption rates 42 to 57% that of ether extracted beef. This resulting decrease of oxygen absorption by

one-half suggests that the oxidation of some non-ether extractable lipid component could be responsible for half of the total oxygen absorption of freeze-dried beef.

Studies of possible oxidative browning pathways in the deterioration of freeze-dried beef. Freeze-dried beef is known to deteriorate in storage in inert gas atmospheres principally by active-aldehyde browning involving some of the metabolic intermediates of carbohydrate metabolism, mainly glucose. In the presence of oxygen the deterioration is more rapid, suggesting that the oxygen absorption noted may be a manifestation of an oxidative pathway of browning. The reductone-dehydroreductone reaction is an example *(5)* of such an oxidative pathway in browning.

The possibilities of oxidative browning were tested by adding to freeze-dried beef those compounds which should accelerate or inhibit oxygen absorption if oxidative browning were operative. Thus, for the acceptance of the reductone hypothesis the reductones listed as additives in Table 5 should give increased oxygen absorption and browning. The reductones expected in unmodified freeze-dried meat would be nitrogenous glucose reductones similar to piperidino-, morpholino-, and dimethylamino-glucose

TABLE 5

The effect of adding possible reactants and inhibitors on the oxidative reaction

Experiment	Classification of additive	Additive	Concentration	Rate of oxygen absorption cu. mm. O_2/g./hr.	$\frac{CO_2 \text{ evolved}}{O_2 \text{ absorbed}}$
1		None		13.0	0.20
	Aldehyde complexing reagents	Sodium bisulfite	1000 p.p.m. SO_2	12.6	0.18
		Semicarbazide	Same molar conc. as SO_2	13.6	0.16
	Sugar	Glucose	1%	13.3	0.18
2		None		10.0	
	Reductones	Piperidino-glucose reductone	1%	9.0	
		Morpholino-glucose reductone	1%	9.1	
		Dimethylamino-glucose reductone	1%	9.5	
	Metabolic intermediates	Glucose	4%	9.7	
		Lactic acid	10%	9.4	
		Glycogen	10%	10.3	
		Fructose-1, 6-diphosphate	1%	6.9	
		Adenosine triphosphate	1%	10.0	

reductones. The results reported in Table 5 do not support the hypothesis that the reductone pathway might be responsible for oxygen absorption.

If another oxidative browning pathway were operative in which carbonyls were precursors, the addition of carbonyl complexing agents should give inhibition. The addition of sodium sulfite or semicarbazide gives no inhibition. This result suggests that carbonyls are not precursors to the oxidative reaction.

If carbohydrate-protein browning were responsible for the oxygen absorption, then the addition of large quantities of metabolic intermediates to the freeze-dried beef should give increased oxygen absorption in the cases of all precursors of the oxidative reaction. Table 5 shows that none of the metabolic intermediates known to be naturally present in beef when added in relatively large concentrations give an increase in the rate of oxygen absorption. Fructose-1,6-diphosphate, for some unknown reason, actually inhibits.

All of these results show that oxygen absorption by stored freeze-dried beef cannot be ascribed to an oxidative pathway of active-carbonyl carbohydrate-amine browning.

The oxidation of the protein fraction. Except for a report on the oxidation of dry hemoglobin *(4)*, the research literature contains practically no information on the oxygen lability of dry proteins. Because it is generally assumed that dry proteins are stable in oxygen, attention was not directed to the protein fraction of freeze-dried beef until the possibilities of unsaturated fat oxidation and oxidative carbonyl-amine browning were evaluated.

To test the possibility that the oxygen absorption of freeze-dried beef was a measure of oxidation of protein or some compound intimately asso-

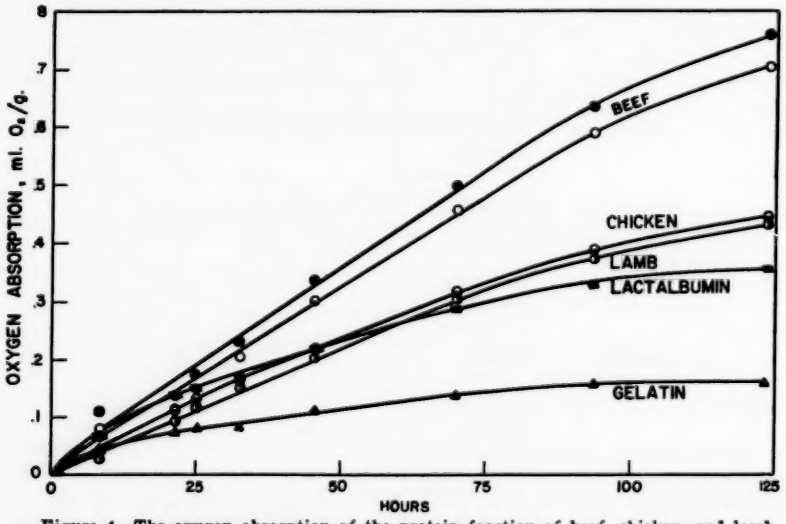

Figure 4. The oxygen absorption of the protein fraction of beef, chicken, and lamb and the proteins, lactalbumin and gelatin.

ciated with it, the oxygen absorption of protein fractionated from two samples of freeze-dried beef was measured. For comparison, the protein fraction from freeze-dried lamb and chicken and the protein, lactalbumin, gelatin, and sodium caseinate, all equilibrated to a water vapor pressure of 0.65 mm. Hg., were included in this study. The results in Figure 4 show by comparison with all available information on the rate of oxygen absorption of freeze-dried beef that the protein fraction oxidizes at a rate sufficient to account for from one-half to all of the oxygen absorption of freeze-dried beef. That the oxidation of the protein fraction is a general deteriorative reaction in freeze-dried meats is shown by the oxidation of the protein fractions from freeze-dried lamb and freeze-dried chicken.

Further, the oxygen absorption of lactalbumin and gelatin suggested that oxygen lability is a general property of proteins. Continuing studies of the oxidation of purified and crystalline proteins support this general suggestion. Oxidation of protein is probably an important reaction in the oxidative deterioration of other high-protein dehydrated foods such as milk and eggs. Oxidation of protein may be an important deteriorative mechanism for a loss of functional and biological properties in freeze-dried blood plasma, protein hormones, enzymes and other biological proteins.

The information available on the oxygen lability and the chemical oxidation of amino acids and proteins in aqueous solutions suggests that the amino acids: cystine, histidine, methionine, tyrosine, and tryptophane would be most oxygen labile *(14, 15, 17, 18)*. Studies of the mechanism of oxidation of dry proteins are continuing.

SUMMARY

During the freeze-drying of beef, oxymyoglobin is deoxygenated to myoglobin. During storage and upon subsequent rehydration, the myoglobin in freeze-dried beef is readily oxidized to metmyoglobin. This oxidation of myoglobin is primarily responsible for the early development of an undesirable brown color during storage of freeze-dried beef.

During storage at 38°C., freeze-dried beef will react chemically with relatively large amounts of atmospheric oxygen. This oxidative reaction is not coupled with the main deteriorative reaction of active carbonyl-amine browning. The oxidation of ether-soluble lipids can, at most, account for 10% of the total oxidative reaction. Oxidation of non-ether-soluble conjugated lipids could account for approximately 50% of the total oxygen absorption. The most unusual and most deteriorative oxidative reaction in freeze-dried beef appears to involve the oxidation of the protein fraction. The oxidation of the protein fraction of freeze-dried beef can account for 50-100% of the total oxygen absorbed.

The main oxidative deterioration may be prevented by storing freeze-dried beef in inert atmospheres or at high vacuum. Storage in inert atmospheres at high vacuum does not prevent the oxidation of myoglobin in freeze-dried meat. Conversion of myoglobin to carboxymyoglobin or nitrosomyoglobin gives a stable meat color. Rehydration of freeze-dried beef in oxygenated water is suggested in order to promote the oxygenation of myoglobin and to suppress its oxidative reaction.

Acknowledgments

The author wishes to express his sincere appreciation to Merle R. Emerson for measurements of fat oxidation, spectral absorption of hematin pigments, and the effect of some chemical additives, to Albert Conroy, Lloyd W. Regier, and George F. Stewart for their assistance and suggestions throughout the course of this study.

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ON THE NATURE OF CELLULOSE FROM CRANBERRY PULP[a]

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Cellulose from a chemical point of view is a polymer of cellobiose. In the plant, however, such homogeneity is the exception. Variable amounts of cellulosans are usually present in addition to "true" cellulose. Analytical procedures *(1, 3)* for cellulose, in woody or nonwoody tissue, yield such a product which upon hydrolysis yields from a trace to 3 to 4% of lignin as the residual fraction. The cellulose from cranberry "cocktail" pulp, however, was found to yield approximately 31% of "apparent" lignin or a residue which was unhydrolyzed by the action of 72% sulfuric acid. A cellulose with such a high content of lignin is without precedent. A further investigation revealed that the high yield was due to the incomplete removal of cutinous or waxy substances. Since these substances are not easily wetted or soluble in aqueous media, they may remain in the plant residue if they are not completely extracted previously by the organic solvents; furthermore, they may shield substances which would otherwise be removed. Since plants contain variable amounts of cutinous-like substances, a conventional procedure for their removal may be inadequate for successful subsequent analyses. The effect of incomplete removal of such substances on the yield of other fractions has not received due consideration *(6)*.

EXPERIMENTAL

Cranberry "cocktail" pulp is the name for the residue remaining after the juice has been removed by pressure. The dried pulp was ground to pass a one-half millimeter sieve and extracted in a Soxhlet extractor with a solution of ethyl alcohol and benzene in the volume ratio 1:2. The extraction was carried on for about 6 hours at the rate of one extraction every 3 minutes. The extract was colorless at the end of this period. After removing the remaining alcohol and benzene with ethyl ether the sample was dried. Pectic materials were removed by extraction with 0.5% ammonium citrate for 24 hours, after which the residue was thoroughly washed with hot water, alcohol, and ether, and then dried. Cellulose was determined on the extracted material by the Bennett *(1)* modification. This procedure among others uses the Mäulé reaction to indicate the presence of lignin. The Mäulé reaction was positive after the first chlorination but was negative after the second, indicating the absence of lignin. The residues were treated as recommended and then analyzed by the 72% sulfuric acid method essentially as recommended by Norman and Jenkins *(3)*. Hydrolysis was allowed to continue over a period of 15 hours in a refrigerator operating at 4°C. The residue obtained after the final hydrolysis was a light brown, coarse, rather fibrous substance, which was easily recovered by filtration.

RESULTS AND DISCUSSION

The results of the analyses of the pulp, to the nearest per cent, on an ash- and moisture-free basis are as follows:

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[a] Contribution No. 1011 from the University of Massachusetts Agricultural Experiment Station, Amherst.

	%
Alcohol-benzene extract	24
Citrate-water extract	17
Sulfite extract	28
Cellulose	31

The cellulose fraction consisted of 69% of cellulose and 31% of "apparent" lignin. The latter fraction included the actual lignin. The sugars in the cellulose hydrolyzate were recovered from the sulfuric acid in the usual manner. The solution was analyzed by paper chromatographic procedures using ethyl acetate, water, and pyridine in the ratio of 2:2:1 as the solvent system *(2)* and phthalate spray *(4)* to locate the sugars. The presence of glucose, arabinose, and xylose was indicated.

A preliminary investigation indicated that at room temperature the residue was insoluble in pyridine, ethanol, benzene, and all solvents used to classify organic compounds; wetting and appreciable solubility occurred in hot 4% sodium hydroxide. Concentrated sulfuric acid eventually charred and hydrolyzed the material. A microscopic examination indicated the presence of the epicarp composed of unimpaired polygonal cells with pits. The material stains with Sudan III and is believed to be chiefly cutinous in nature. The chemical behavior of some of the substance removed with hot alkali is similar to that of ursolic acid *(5)*. In general, the extraction of fats, waxes, cutins, etc., from plant material is carried out for periods ranging from 2 to 30 hours; the shorter periods are more widely used. Unless the extraction is complete, these substances may greatly affect the action of subsequent solvents and hence the yield of certain groups. In this case, the thick cutinous deposit was not completely removed at the proper time, thus causing an increase in the yield of cellulose and lignin.

CONCLUSIONS

Conventional chemical procedures to remove cutinous substances from plant material are sometimes inadequate. Incomplete removal of these substances will enhance the yields of cellulose and lignin to levels considerably beyond those which actually exist.

Acknowledgment

The author is indebted to the Department of Botany for the microscopic interpretation.

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EFFECT OF MOIST AND DRY HEAT COOKING ON VITAMIN RETENTION IN MEAT FROM BEEF ANIMALS OF DIFFERENT LEVELS OF FLESHING[a]

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(Manuscript received September 1, 1955)

Thiamine and niacin retention in beef after cooking has been studied in various cuts cooked in various ways. Weisman and Elvehjem *(11)* working with fried steaks and oven roasts and Tucker, Hinman, and Halliday *(10)* working with fried, broiled and braised beef attributed the higher retentions in fried steaks to the shorter cooking times. Cover, McLaren, and Pearson *(4)* obtained higher thiamine retention in rare than in well-done roasts. The cooking time was shorter for the rare than for the well-done roasts, but the internal meat temperature was lower in rare roasts. Cover, Dilsaver, Hays, and Smith *(3)* and Mayfield and Hedrick *(8)* reported that roasts cooked for a longer time at low oven temperatures retained more thiamine than those cooked at high oven temperatures for a shorter time. Moreover, Cover and Dilsaver *(1)* using cubes of brisket stewed by 12 different methods, including variations in browning, temperature of cooking, and amount of water, found that browning appeared to be the one cooking variable which significantly lowered thiamine retention even though temperatures of cooking varied from 93° to 121°C. and cooking times from 20 to 180 minutes. Hood, Thompson, and Mirone *(7)* found no significant difference in thiamine retention between moist and dry heat methods either in thin cuts or in thick cuts, although the cooking times were shorter for the moist heat methods.

Niacin, unlike thiamine, is not heat labile. Different methods of cooking have shown variations in niacin retention in the meat but most of the remaining niacin has been found in the drippings *(2, 9)*.

This study was designed to obtain further information on the losses of thiamine and niacin during cooking of thin and thick cuts of beef by moist and dry heat methods.

EXPERIMENTAL PROCEDURES

Additional paired cuts for this study were obtained from 4 animals used by Cover and Shrode *(5)* in the study of palatability scores and shear force values. Steaks ¾-inch thick were obtained from loin and bottom round. The bottom round included *biceps femoris* and *semitendinosus* muscles. Roasts and pot roasts were cut 2½ inches thick, from the standing rib only. The cut from one side of the animal was analyzed raw and the one from the other after cooking. Both the raw and cooked cuts were of equal thickness. The raw and cooked samples from the same position within an animal were alternated between the right and left sides. Before cooking the excess fat was trimmed from the meat so that sampling for vitamin analyses could be done more accurately.

[a] This study was supported in part by the U. S. Department of Agriculture through a contract sponsored by the Human Nutrition Research Branch, Agricultural Research Service.

However, this procedure made comparison of the ether extract values unreliable as a measure of fatness for pasture vs. feedlot animals.

Methods of cooking were those described by Cover and Shrode *(5)*. The meat was weighed before and after cooking. The cooked meat was cooled in a covered container. The bone was removed and its weight recorded. Weights of boneless meat before and after cooking were calculated. The meat (lean and fat) was ground through the power grinder—twice through the coarse and three times through the fine plate.

The methods for moisture and ether extract were essentially those of AOAC and for thiamine and niacin essentially those described in *Methods of Vitamin Assay of the Association of Vitamin Chemists.*

RESULTS

Weights, weight losses, and cooking times. Loin steaks for analysis were posterior to those used for palatability tests by Cover and Shrode *(5)* and therefore slightly heavier. The rib roasts were anterior to those used for palatability and therefore considerably heavier. The bottom round steaks were similar in weight for the two studies.

Weight losses during cooking were similar for the two methods of cooking in bottom round steaks (Table 1), but were somewhat greater for broiled loin than for braised loin steaks and for oven roasts than for pot roasts.

TABLE 1

Weights, weight losses, and cooking times for cuts of beef used in vitamin tests

Method of cooking	Animal number[1]	Number of cuts averaged	Weight of raw meat grams	Weight of bone grams	Weight losses during cooking %	Cooking time minutes per pound
			Loin steaks			
Broiled	III	3	325	41	42	50
	III A	3	371	40	46	43
	IV	3	416	61	36	39
	IV A	3	355	54	37	46
Braised	III	3	412	61	35	49
	III A	3	430	49	31	49
	IV	3	481	87	31	43
	IV A	3	399	75	33	53
			Bottom round steaks			
Broiled	III	2	277	....	42	56
	III A	2	287	....	44	53
	IV	2	463	....	41	34
	IV A	2	360	....	41	45
Braised	III	2	267	....	42	77
	III A	2	303	....	42	68
	IV	2	474	....	40	44
	IV A	2	389	....	40	53
			Rib roasts and pot roasts			
Oven roast	III	2	1599	248	38	83
	III A	2	1596	219	35	73
	IV	2	1809	232	35	69
	IV A	2	1995	229	31	59
Pot roast	III	2	1390	266	26	26
	III A	2	1413	215	26	25
	IV	2	1747	215	26	23
	IV A	2	1799	170	23	22

[1] Animals III and III A were slaughtered off pasture, IV and IV A out of the feedlot.

TABLE 2

Average thiamine content and retention of thin and thick cuts of beef

Method of cooking	Animal number	Number of cuts averaged	Raw		Cooked			
			Thiamine content		Thiamine content		Thiamine retention[1]	
			Moist basis γ/g.	Dry fat-free basis γ/g.	Moist basis γ/g.	Dry fat-free basis γ/g.	In meat %	In meat and broth %
Loin steaks								
Broiled	III	3	0.74	3.95	0.81	2.71	58	....
	III A	3	0.79	4.57	0.78	2.59	48	....
	IV	3	0.83	4.50	0.90	3.18	62	....
	IV A	3	0.73	3.90	0.75	2.57	58	....
							Mean 57	
Braised	III	3	0.81	4.47	0.42	1.68	31	58
	III A	3	0.80	4.87	0.41	1.85	33	58
	IV	3	0.86	4.68	0.55	2.12	39	65
	IV A	3	0.74	3.90	0.43	1.56	34	59
							Mean 34	Mean 60
Bottom round steaks								
Broiled	III	2	0.92	4.34	0.97	2.80	62	....
	III A	2	0.96	4.32	1.05	2.81	61	....
	IV	2	0.95	4.61	1.01	3.15	63	....
	IV A	2	0.59	2.92	0.56	1.74	57	....
							Mean 61	
Braised	III	2	0.92	4.32	0.66	1.94	42	65
	III A	2	0.97	4.42	0.61	1.70	37	65
	IV	2	0.95	4.67	0.54	1.76	35	65
	IV A	2	0.61	2.95	0.40	1.28	39	66
							Mean 38	Mean 65
Rib roasts and pot roasts								
Oven roast	III	2	0.70	3.64	0.43	1.42	35	....
	III A	2	0.74	4.06	0.50	1.81	43	....
	IV	2	0.71	4.20	0.47	1.87	40	....
	IV A	2	0.57	3.66	0.40	1.94	46	....
							Mean 41	
Pot roast	III	2	0.64	3.46	0.43	1.74	47	64
	III A	2	0.73	3.87	0.43	1.77	41	58
	IV	2	0.69	4.13	0.47	2.18	48	64
	IV A	2	0.53	3.51	0.43	2.27	60	70
							Mean 49	Mean 64

[1] Thiamine retention was calculated according to the following formula:

$$\frac{\text{Wt. in g. of boneless meat after cooking} \times \text{content per g. of cooked meat}}{\text{Wt. in g. of boneless meat before cooking} \times \text{content per g. of raw meat}} \times 100 = \% \text{ retention in meat}$$

$$\frac{\text{Total content in broth} \times 100}{\text{Wt. in g. of boneless meat before cooking} \times \text{content per g. of raw meat}} = \% \text{ retention in broth}$$

Thiamine content and retention. There was considerable variability among the individual animals in the thiamine content of similar cuts but no consistent difference in thiamine content or retention between the animals (III and III A) which were slaughtered off pasture and those (IV and IV A) slaughtered out of the feedlot. (Table 2).

Thiamine retention in steaks was considerably higher in the broiled than in the braised of both loin and bottom round steaks. The difference was highly significant statistically. The meat of the pot roasts retained somewhat more thiamine than that of the oven roasts. The difference was significant statistically. Thus, one dry heat

method (broiling) was more favorable and another (oven roasting) less favorable for thiamine retention than the corresponding moist heat methods. This contradiction leads to the conclusion that factors other than moist and dry heat cooking are involved. Other possible factors will be presented in the discussion.

Niacin content and retention. There was no indication of a consistent difference in either niacin content or retention between the animals slaughtered off pasture (III and III A) and those out of the feedlot (IV and IV A). (Table 3).

TABLE 3

Average niacin content and retention of thin and thick cuts of beef

Method of cooking	Animal number	Number of cuts averaged	Raw		Cooked			
			Niacin content		Niacin content		Niacin retention[1]	
			Moist basis γ/g.	Dry fat-free basis γ/g.	Moist basis γ/g.	Dry fat-free basis γ/g.	In meat %	In meat and broth %
Loin steaks								
Broiled	III	3	43	231	56	185	68	....
	III A	3	34	195	47	157	68	....
	IV	3	34	186	46	162	77	....
	IV A	3	38	205	51	173	75	....
							Mean 72	
Braised	III	3	42	232	29	115	41	76
	III A	3	32	193	25	111	51	91
	IV	3	35	189	29	110	51	94
	IV A	3	36	189	29	107	49	94
							Mean 48	Mean 89
Bottom round steaks								
Broiled	III	2	44	177	43	145	78	....
	III A	2	40	178	52	139	73	....
	IV	2	37	182	46	144	73	....
	IV A	2	39	193	46	144	70	....
							Mean 74	
Braised	III	2	39	184	34	99	45	88
	III A	2	39	177	35	98	53	94
	IV	2	39	189	32	105	51	92
	IV A	2	41	199	32	103	47	86
							Mean 49	Mean 90
Rib roasts and pot roasts								
Oven roast	III	2	29	152	42	137	80	....
	III A	2	29	159	33	121	70	....
	IV	2	28	168	34	137	72	....
	IV A	2	27	175	26	127	64	....
							Mean 72	
Pot roast	III	2	29	158	26	103	61	99
	III A	2	31	167	27	113	60	96
	IV	2	30	179	23	108	55	87
	IV A	2	28	187	27	143	73	106
							Mean 62	Mean 97

[1] **Niacin retention was calculated according to the following formula:**

$$\frac{\text{Wt. in g. of boneless meat after cooking} \times \text{content per g. of cooked meat}}{\text{Wt. in g. of boneless meat before cooking} \times \text{content per g. of raw meat}} \times 100 = \% \text{ retention in meat}$$

$$\frac{\text{Total content in broth} \times 100}{\text{Wt. in g. of boneless meat before cooking} \times \text{content per g. of raw meat}} = \% \text{ retention in broth}$$

Niacin retention in the broiled was considerably higher than in the braised steaks. Most of the niacin lost from the meat of braised steaks was found in the broth. Rib oven roasts retained more niacin in the meat than did pot roasts but when pot roasts and their broth were considered together most of the niacin present in the raw meat was accounted for. Niacin is not heat labile and no destruction was expected.

DISCUSSION

Waisman and Elvehjem *(11)* and Tucker, Hinman, and Halliday *(10)* suggested that a short cooking time even at high temperature favors thiamine retention. Several comparisons may be made from the data in Table 2

TABLE 4

Thiamine in the surface layer of steaks

Animal number and cut number	Method of cooking	Thiamine content		Thiamine retention			
		Brown layer γ/g.	Interior γ/g.	Brown layer %	Interior %	Broth %	Total %
Top round							
IV							
6	Broiled	1.78	1.03	15	50	[1]	65[2]
7	Braised	0.54	0.62	4	32	23	59
IV A							
6	Broiled	1.59	0.90	13	52	[1]	65[2]
7	Braised	0.46	0.59	3	38	31	72
Bottom round							
IV A							
9	Broiled	2.25	1.04	12	54	[1]	66[2]
10	Braised	0.67	0.63	3	34	30	67
IV							
9	Broiled	2.61	1.17	12	55	[1]	67[2]
10	(Cut not available for cooking)						

[1] No analyses were made on drippings from broiled steaks because quantitative transfer seemed unlikely.
[2] This figure does not include thiamine in drippings and so "total" is for meat only.

to test this hypothesis. Broiled steaks submitted to high cooking temperatures for a short time had higher thiamine retentions than did oven roasts at lower cooking temperatures but much longer cooking times. These data appear to be the only ones that follow the trend suggested.

As between oven and pot roasts, thiamine retention in oven roasts was somewhat lower than in pot roasts. Not only was the time of cooking much longer for the oven roasts but the cooking temperature was higher also; yet the difference in thiamine retention was not very great.

Comparison of braised steaks with pot roasts showed the retentions were higher in the meat of pot roasts with longer cooking times but the same cooking temperature. However, when the braised steaks and pot roasts were compared on the basis of the totals of meat and broth (Table 2), the braised steaks and the pot roasts retained about the same percentage of thiamine. This indicated little if any difference in destruction of thiamine by the two moist heat methods of cooking even with the cooking time by one method approximately double that of the other.

In the comparison of broiled with braised steaks, broiled steaks show more thiamine retention than braised steaks, but if the broth is used the total retention in braised steaks is brought to the level of the meat of broiled steaks. The drippings from the broiled steaks were not analyzed because quantitative transfer seemed unlikely. Therefore, no direct comparison is possible between the total retentions of broiled and braised steaks. However, it may be noted that part of the drippings from steaks broiled by this method are in the form of jelly. This jelly might be expected to retain some thiamine. In that case total retention for broiled steaks should be higher than total retention for braised steaks.

Since thiamine and niacin are water soluble vitamins, the retention of these vitamins may be related to the kind of moisture lost during cooking. This loss is of two kinds, drippings and evaporation. Drippings would carry the vitamins away with them but evaporation would not. Evaporation would take place in broiled steaks and oven roasts but not in braised steaks and pot roasts. During broiling, some protein material (probably water soluble), collects and coagulates on the surface of the steak. Perhaps water soluble vitamins are left in this film as the moisture in which they were dissolved is evaporated. When the steak is turned the new surface loses moisture so rapidly that the internal temperature of the ¾-inch steak drops several degrees. Such rapid evaporation from the surface may cool it enough to prevent excessive destruction of thiamine as long as the surface remains moist—as it does in steaks but not in roasts. This surface film was not observed on braised steaks. It is inferred that the condensing steam washed it off as it formed.

To get an indication of whether more thiamine was actually retained on the surface of broiled than of braised steaks, a few pairs of steaks from two of the animals were subjected to special treatment. One steak of each pair was analyzed raw and the other after cooking. Cooking was by broiling in one pair and by braising in the other pair. After cooking and cooling (in covered containers), the film and browned surfaces were removed from each cooked steak. The brown layer, interior, and broth were analyzed separately (Table 4). The thiamine content was considerably higher per gram in the brown layer of broiled steaks than in the interior. This was not true of the braised steaks. The brown layer in broiled steaks contained from 12 to 15% of the thiamine present originally but in braised steaks it contained only 3 or 4%. Thus thiamine appeared to be more concentrated in the surface layer of broiled but not of braised steaks. This, however, is evidently not the whole story. The interior of the broiled steaks had more thiamine than that of the braised. Leaching of thiamine from the interior of the meat may be an explanation. Another possible explanation is that in braised steaks the internal temperature reached 100°C. about 25 minutes before the end of the cooking period; in broiled steaks the maximum internal temperature was not more than 80°C. The higher internal temperature in braised steaks may have caused the greater destruction of thiamine.

Thiamine and niacin retentions in the meat may be related to the moisture retained. In broiled and braised steaks, moisture retention was about 45%, but thiamine and niacin retentions in broiled steaks were considerably

TABLE 5

Cooking conditions and thiamine, niacin and moisture retention in thin and thick cuts of beef

Method of cooking	Number of cuts averaged	Temperature of cooking °C	Final meat temp. °C	Time of cooking minutes	Thiamine retained in meat %	Niacin retained in meat %	Moisture retained in meat %
Broiled steaks	20	200	[1]	35	59	73	43
Braised steaks	20	100	100	45	36	48	45
Oven roasts	8	150	80	270	41	72	45
Pot roasts	8	100	92	81	49	62	55

[1] Thermometer readings dropped when steak was turned and seldom reached 80°C. even though meat was well-done.

higher than this (Table 5), possibly indicating that some of each vitamin was left behind on the surface of the meat after evaporation. In braised steaks, niacin retention in the meat was not much higher than 45%, but thiamine retention was somewhat lower. The high internal temperature of the braised steaks would favor thiamine destruction but would not affect niacin.

In rib oven roasts moisture retention was 45% also, but evaporation took place over a longer period and the surface became much drier than in steaks. In this case the surface temperature must have been higher than in steaks, favoring destruction of most if not all of the thiamine on the surface and probably in the adjacent underlying areas also. Actually, thiamine retention in oven roasts was lower than in broiled steaks and almost as low as in the meat of braised steaks, but niacin retentions in oven roasts were as high as in broiled steaks. Moisture retention in pot roasts was the highest for any of these methods of cooking, and the thiamine and niacin retentions in the meat were also the highest of any moist heat method.

When considered in respect to the daily allowance of 1.5 mg. of thiamine for 3000 calories recommended by the Food and Nutrition Board *(6)*, the amount supplied by an entire steak after broiling or braising would be respectively about 10% vs. about 6% of the daily allowance. If the allowance for niacin is taken as 15 mg., the amount supplied in a steak after broiling or braising would be respectively about 60% vs. 40% of the daily allowance.

The effect of these two methods of cooking steak on the thiamine intake may be of doubtful practical importance unless the food supply is severely limited. On the other hand, beef is such a good source of niacin that the method of cooking steaks could have a practical effect on the niacin intake.

For the oven roasts and pot roasts, 4 ounces of raw meat were used as a serving. On this basis the two methods of cooking differed by 3% or less of the recommended daily allowances of thiamine and niacin. It is evident that the method of cooking thick cuts is of no great practical importance in respect to intake of these vitamins.

CONCLUSIONS

Thiamine and niacin retentions were higher in the meat of broiled than in the meat of braised steaks. Braised steaks together with the broth, how-

ever, contained as much thiamine and more niacin than was found in the meat alone of broiled steaks.

The meat of the oven roasts contained somewhat less thiamine but more niacin than the meat of pot roasts. But when the meat and broth of pot roasts were considered together they retained more thiamine and niacin than was found in the meat alone of the oven roasts.

Some evidence is presented that thiamine and niacin may be held on the surface of the meat during broiling but not during braising.

Evaporation from the surface of the meat during cooking by dry heat methods or washing of the surface by condensing steam during cooking by moist heat methods may be important factors affecting retention of thiamine and niacin. Internal temperature of the meat also may be an important factor in thiamine retention. Thus, it is impossible to make generalizations relating the thiamine retention of home cooked beef to the cooking temperature, to the cooking time, or to the moisture content of the cooked meat.

No evidence was obtained that the pasture and feedlot animals differed in either content or retention of thiamine and niacin.

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THE HYDROLYSIS OF BEEF PROTEINS BY VARIOUS PROTEOLYTIC ENZYMES[a]

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Recently the use of proteolytic enzymes to tenderize meats has increased tremendously. The enzyme papain has been used extensively in commercial meat tenderizers, but many other enzymes also possess the prerequisite proteolytic activity for meat tenderization. Because of the wide variety of proteolytic enzymes from plant, animal, and microbiological sources, which appear suitable and have been suggested as meat tenderizers, a comparison is desirable.

As an integral part of the investigation on papain and meat tenderization being conducted in this laboratory, the relative merits of different proteolytic enzymes[b] were investigated by digesting rehydrated, ground, freeze-dried *biceps femoris* muscle of beef with the various enzymes and then fractionating the component protein fractions of meat by extraction and precipitation techniques. In order to evaluate the extent and mechanism of digestion, the soluble-nitrogen fraction was separated into soluble-protein nitrogen and non-protein nitrogen, and the insoluble-protein fraction was separated into collagen and elastin. The degree of enzymatic digestion of each fraction and sub-fraction was determined by Kjeldahl nitrogen determinations or hydroxyproline determinations.

Very little has been written on the relative activities of different proteolytic enzymes on meat. Essentially, the only information on this subject comes from Gottschall and Kies *(5)* and Hay, Harrison, and Vail *(6)*, who have reported investigations on meat digestion and tenderization by papain and a commercial papain-constituted meat tenderizer, respectively.

Despite the recent investigations by Lampitt, Baker, and Brown *(10, 11)* on the relative extractive ability of dilute hydrochloric acid and sodium hydroxide solutions in extracting soluble meat proteins, it appeared desirable to extend their investigation to include two additional reagents, 0.5% potassium chloride and 20% urea, which are also used for extracting soluble meat proteins. Soluble proteins have been extracted with urea solutions by Neuman and Logan *(15)* and Beck and Schormüller *(3)*, while 0.5% potassium chloride solution has been used as an extraction medium by investigators at this laboratory in dehydrated meat studies. The sodium hydroxide extraction procedure is essentially that introduced by Lowry, Gilligam, and Katersky *(12)* and subsequently employed by Prudent *(16)*, Husaini, Deatherage, Kunkle, and Draudt *(7, 8)* and Baker, Lampitt, and Brown *(10, 11)*. In comparing extractions with hydrochloric acid and sodium hydroxide, the last group of investigators found sodium hydroxide

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[b] The enzymes used in this investigation are papain, bromelin, ficin, trypsin, pepsin, Rhozyme P-11, Protease 15, Rhozyme A-4, and a commercial meat tenderizer containing papain.

to be superior to hydrochloric acid. The Kjeldahl nitrogen and the hydroxyproline determinations were used as criteria of the ability of the different extraction procedures tested to separate the insoluble from the soluble protein.

EXPERIMENTAL

Materials. The proteolytic enzymes Rhozyme P-11, Protease 15, and Rhozyme A-4 were obtained from the Rohm and Haas Company; pepsin and papain from Nutritional Biochemical Corporation; ficin from Mann Research Laboratories; trypsin from General Biochemicals Incorporated; and bromelin from the Pineapple Research Institute.

P-dimethylaminobenzaldehyde (Eastman Grade organic chemical) was recrystallized twice from ethanol according to the directions of Neuman and Logan *(14)*. N-propanol was also an Eastman Grade organic chemical, and the fraction distilling between 96° and 97° was used. The 5% p-dimethylaminobenzaldehyde reagent was prepared by dissolving 5 g. of p-dimethylaminobenzaldehyde in n-propanol and diluting to 100 ml.

The stock solution of pig hemoglobin substrate was made from defibrinated, fresh hog blood. The red blood cells were centrifuged and washed three times with physiological saline solution. The cells were then lysed with distilled water, giving a stock solution 4.2×10^{-3} M in hemoglobin. Hemoglobin was assayed as cyanomethemoglobin. The urea-denatured hemoglobin substrate was prepared according to the procedure of Anson *(1)*. To 79 ml. of distilled water were added 4 ml. of 2 N sodium hydroxide, 36 g. of urea, and 8 ml. of stock hemoglobin solution in that order. The solution was mixed and kept at room temperature for 30 to 60 min. to denature hemoglobin. Finally, 10 ml. of 1 M potassium dihydrogen phosphate, 4 g. of urea, and 2 mg. of merthiolate were added. The final solution, 2.58×10^{-4} M in hemoglobin, was stored at 5°C.

The hemoglobin substrate used in the assay of pepsin was also prepared according to the procedure of Anson *(1)*. To 4 ml. of the stock hemoglobin solution was added 1 ml. of 0.3 N hydrochloric acid. The resulting solution (pH 1.6) was stored at 5°C. and used within the prescribed time interval.

The ground-meat substrate used in evaluating the relative activities of different enzymes was prepared by first freeze-drying the *biceps femoris* muscle of beef and then grinding it in a Wiley Mill, using a No. 10 screen. Moisture determinations of the final product indicated 3% moisture on a dry-weight basis as determined by drying over magnesium perchlorate in a vacuum desiccator at room temperature.

Methods. *Assay of enzymes.* All of the enzymes were assayed according to procedures described by Anson *(1)*. The optimum conditions for maximum enzymatic activity were approximated as closely as possible for all enzymes and are tabulated in Table 1. In the assay, 1 ml. of enzyme solution was added to 5 ml. of substrate which was at the desired temperature of assay. The digestion mixture was mixed and allowed to react for 10 min. The reaction was stopped by adding 10 ml. of 4% trichloroacetic acid. The resulting precipitate was centrifuged and the supernatant removed for read-

TABLE 1

The hemoglobin assay of various enzymes

	Concentration %	Urea denatured Hb substrate	Incubation temperature (°C.)	pH	Activation by cyanide	Absorbance at 277 mμ
Bromelin	0.05	Yes	40°	7.5	Yes	0.615
Papain	0.05	Yes	40°	7.5	Yes	0.531
Ficin	0.05	Yes	40°	7.5	Yes	0.830
Trypsin	0.10	Yes	25°	7.5	No	0.103
Pepsin	0.05	No	25°	1.6	No	1.050
Rhozyme P-11	0.05	Yes	40°	7.5	No	0.341
Rhozyme A-4	0.05	Yes	40°	7.5	No	0.147
Protease 15	0.05	Yes	40°	7.5	Yes	0.162
Commercial meat tenderizer	1.00	Yes	40°	7.5	No	0.059
Tyrosine (2×10^{-4}M)						0.260

ings of absorbance at 277 mμ on a Beckman Model DU Spectrophotometer. A blank was used in which trichloroacetic acid was added before the enzyme was introduced.

A comparison of three reagents used in the fractionation of meat proteins. Two-gram samples of ground, freeze-dried *biceps femoris* muscle of beef were rehydrated with 10 ml. of water for 30 min. at room temperature. The rehydrated samples were then extracted with the following reagents: 0.10 N sodium hydroxide, 0.5% potassium chloride, and 20% urea solutions. The extraction procedures for the three extraction media were identical; this procedure is described in detail using sodium hydroxide as the model.

Fifty ml. of 0.10 N sodium hydroxide solution was added to each sample and allowed to stand 16 hrs. The mixture was then centrifuged and the supernatant decanted and saved for combination with three subsequent extraction liquors. The residue was again extracted with 25 ml. of 0.10 N sodium hydroxide for 4 hrs., and the resulting mixture was centrifuged and the supernatant removed. Fifteen ml. of water and one drop of 0.1% phenol red were added to the residue, and the resulting mixture was titrated to neutrality. Vigorous stirring was necessary during the titration in order to allow diffusion of sodium hydroxide from the meat particles. The neutralized mixture was centrifuged, the supernatant decanted, and the residue again extracted for 2 hrs. with 25 ml. of a 3:1 mixture of 95% ethanol and ether. This mixture was centrifuged, and the supernatant was removed and pooled with all previous extraction liquors obtained up to this point. The remaining residue, representing the insoluble proteins of meat, was extracted with 25 ml. of anhydrous ether for 30 min. The mixture was centrifuged, the ether extract was discarded, and the residue representing the insoluble-protein fraction was air-dried.

The first 4 extracts for each sample were combined and made up to a definite volume. One-tenth of this volume was removed for soluble nitrogen determinations.

Collagen was separated from elastin by autoclaving which conveniently transforms collagen into readily soluble gelatin. The air-dried, insoluble-protein fraction representing collagen and elastin was autoclaved at a steam pressure of 15 p.s.i. for two 3-hr. periods using 25 ml. of water in each period of extraction. The gelatin solutions were removed and the remaining residue was washed once with 25 ml. of water. The gelatin extracts and wash solution were combined, and the nitrogen content of the combined extracts was obtained by a Kjeldahl nitrogen determination. When collagen content was determined by hydroxyproline determinations, the combined extracts and wash solution were reduced in volume to approximately 5 ml. on a steam bath and transferred quantitatively to a test tube with the aid of 5 ml. of concentrated hydrochloric acid. The test tubes were then sealed and autoclaved with subsequent samples.

Considerable residual matter remained even after the removal of collagen, especially in the samples extracted with potassium chloride and urea. Since this extraneous matter would naturally affect the results of elastin determinations, all of the residues were subjected to further extraction with 25 ml. of 0.10 N sodium hydroxide for 30 min. in a boiling water bath. This treatment effectively removed the greater part of the previously insoluble protein. The resulting mixture was cooled and centrifuged. The supernatant was discarded and the residue was transferred to a Kjeldahl flask for nitrogen determination or to a test tube for hydroxyproline determination. In the latter sample, 10 ml. of 6 N hydrochloric acid was used in the transfer. The tube was sealed and autoclaved.

Collagen, elastin, and standard hydroxyproline samples were autoclaved together for a period of 6 hrs. at 15 p.s.i. The vials were cooled and broken. The contents were transferred into flasks and the hydrochloric acid digests were neutralized with sodium hydroxide. The neutralized digests representing hydroxyproline from collagen and elastin samples were diluted to 200 ml. and 100 ml., respectively. The standard solutions were also neutralized and diluted to give standards containing 5, 10, 15, and 20γ of hydroxyproline per ml.

Determinations of the hydroxyproline content of the digests were made according to a modified Neuman and Logan procedure *(13)*. In order to correct for the degradation of hydroxyproline during acid hydrolysis, a calibration curve was made with the autoclaved standards. The hydroxyproline content of the samples was converted to collagen and elastin content by the factors 7.02 *(13)* and 52.3 *(15)*, respectively. The

TABLE 2

A comparison of three methods of extraction as determined by Kjeldahl nitrogen and hydroxyproline determinations.[1]

Extraction method	Kjeldahl determination			Hydroxyproline determination	
	% Nitrogen extracted	% Collagen	% Elastin	% Collagen	% Elastin
0.10 M sodium hydroxide	11.43	2.93	0.88	2.68	0.96
0.5% potassium chloride	3.90	12.81	0.59	2.91	0.70
20% urea	.....	23.88	0.54	2.76	0.79

[1] All figures are reported as averages of three readings.

nitrogen content of samples was converted into collagen and elastin content by the factors 5.38 *(4)* and 5.85 *(19)*, respectively.

Hydrolysis of beef proteins measured by the sodium hydroxide extraction procedure. On the basis of the results obtained in the previous investigation (Table 2), the extraction procedure using 0.10 N sodium hydroxide was selected for the following investigation of the relative activities of various enzymes on rehydrated, freeze-dried meat. However, in this study, fractionation was extended to include the total soluble nitrogenous compounds, soluble protein, non-protein nitrogenous compounds, collagen, and elastin. Collagen and elastin were determined either by their hydroxyproline content or Kjeldahl nitrogen. The extraction procedure is very similar to the one described previously; nevertheless, it differs enough to warrant a detailed description.

Triplicate 5-g. samples of ground, freeze-dried *biceps femoris* muscle of beef were rehydrated with 20 ml. of water at room temperature for 30 min. and 10 ml. of 1% enzyme solutions was then added. In the case of trypsin, 1 ml. of 2% enzyme solution was added. The enzyme and beef substrate were mixed well to insure uniform distribution of the enzyme and were incubated in a 60°C. water bath for 1 hr. The temperature of 60°C. was chosen because extensive studies of the effect of temperature *(13)* showed that papain gave maximum hydrolysis of *biceps femoris* at this temperature. It was desirable to compare the other proteolytic enzymes with papain at 60°C. After 1 hr. of digestion, 100 ml. of 0.10 N sodium hydroxide was added to stop the reaction and to begin the extraction process. After 16 hours of extraction, the mixture was then centrifuged, the supernatant removed, and the residue again extracted with 50 ml. of 0.10 N sodium hydroxide for a period of 2 hrs. This extraction was repeated with another 50 ml. of 0.10 N sodium hydroxide for 2 hrs. After the supernatant was removed from the last sodium hydroxide extraction, 35 ml. of water and 1 drop of 0.1% phenol red were added to the residue and the resulting mixture was titrated to neutrality. During this process, care was taken to allow enough time for the diffusion of sodium hydroxide from the meat particles. The neutralized mixture was centrifuged and the supernatant decanted and saved. The residue was again extracted for 30 min. with 50 ml. of a 3:1 mixture of 95% ethanol and ether. The mixture was then centrifuged, and the supernatant was combined with all previous extracts from the same samples. This solution, making up the soluble-nitrogen fraction, consisted of the supernatant from three sodium hydroxide extractions, one aqueous wash, and an alcohol-ether extraction.

The residue was extracted with 50 ml. of diethyl ether for 30 min. The mixture was centrifuged, the ether discarded, and the residue which represented the insoluble-protein fraction was air-dried and saved for elastin and collagen determinations.

The extract representing the soluble-nitrogen fraction of *biceps femoris* was diluted to 500 ml. A 50-ml. aliquot was removed from each sample for total soluble-nitrogen determination. To another 100-ml. aliquot was added an equal volume of 20% trichloroacetic acid. The trichloroacetic acid precipitate was filtered and washed with 40 ml. of water. Kjeldahl nitrogen determinations were made on both the precipitate and the filtrate, which represented soluble-protein nitrogen and non-protein nitrogen, respectively.

The separation of collagen from elastin was carried out as before. The collagen samples were analyzed for hydroxyproline and nitrogen content as follows. After

hydrolysis with hydrochloric acid, subsequent neutralization, and one-hundredfold dilution, 1 ml. of each sample was removed for a hydroxyproline determination, and the remaining 99 ml. was used for a nitrogen determination.

The nitrogen and hydroxyproline contents of elastin were determined in the same manner.

RESULTS AND DISCUSSION

Three different extraction methods were studied in order to evaluate their applicability to future studies of the effect of various proteolytic enzymes on component fractions of muscle tissue. The results of this investigation are given in Table 2. Obviously, the greatest extraction of soluble protein is obtained with sodium hydroxide. The close agreement between the values for collagen and elastin, as determined by sodium hydroxide extraction and the hydroxyproline method, is one of the main criteria for acceptance of sodium hydroxide extraction. Although this method of extraction possesses obvious disadvantages stemming from sodium hydroxide's slow hydrolysis of proteins, it nevertheless gave the most precise measurements because of the clean separation of protein components. The slight erosion of insoluble protein resulting from the hydrolytic power of 0.10 N sodium hydroxide was not found to be critical in the following studies in which only the relative hydrolyses of different protein components by various enzymes were measured.

An investigation was made to evaluate the ability of various enzyme preparations to hydrolyze different protein components of muscle. For this investigation the sodium hydroxide extraction procedure was selected. The results are given in Tables 3 and 4. In an evaluation of the results, it should be observed that although a definite concentration of each enzyme was used (see Methods), no index of the purity of the enzymes or the enzyme content of the commercial meat tenderizer was available. To compensate for this lack of hemoglobin assay of each enzyme used is given in Table 1. The greatest change among the various protein fractions can be found in the transformation of soluble-protein nitrogen to non-protein nitrogen (Table 3). This change represents the hydrolysis of soluble muscle fiber proteins, mainly actomyosin, to peptides and amino acids by the proteolytic enzymes. In this respect, greatest proteolytic activity was exhibited by the samples of ficin, bromelin, and trypsin, followed by papain and Rhozyme P-11.

Because of the importance of the hydrolysis of muscle-fiber proteins in meat tenderization catalyzed by added proteolytic enzymes, it is interesting to compare this activity of the various enzymes on the basis of the hemoglobin assay as shown in Table 5. In calculating the relative hydrolysis of muscle proteins the increase in non-protein nitrogen catalyzed by each enzyme was divided by the activity of that enzyme in the hemoglobin assay. Adjustments were made for concentration differences, and the activities of pepsin and trypsin in the hemoglobin assay were extrapolated from 25°C. to 40°C., using the corresponding activation energies of 15.4 kcal./mole and 17.7 kcal./mole *(17)*.

In this comparison the commercial meat tenderizer containing small amounts of papain gives a much higher relative hydrolysis than concen-

TABLE 3

Per cent nitrogen of different meat fractions obtained from the digestion of freeze-dried biceps femoris muscle with various proteolytic enzymes.[1]

	Proteolytic enzymes									
	Papain	Rhozyme P-11	Rhozyme P-15	Protease A-4	Commercial meat tenderizer	Bromelin	Ficin	Pepsin	Trypsin	Control
Total soluble nitrogenous compounds	11.85	12.26	11.79	11.80	11.64	12.23	12.34	10.97	12.13	11.29
Soluble protein nitrogen	3.54	4.03	8.09	7.24	8.36	1.97	0.86	8.21	2.40	9.44
Non-protein nitrogen	7.94	7.87	3.55	4.39	3.08	10.32	11.38	2.84	9.93	1.98
Elastin nitrogen	0.088	0.135	0.191	0.186	0.399					0.268
Collagen nitrogen	0.054	0.044	0.292	0.239	0.306	0.019	0.029	0.568	0.037	0.443

[1] Per cent nitrogen are reported as averages of three determinations.
Digestion was carried out for one hour at 60°C.

TABLE 4

The collagen and elastin content of the enzymatic digest of lyophilized biceps femoris muscle as determined by their hydroxyproline content

Enzymes	% Collagen	% Elastin
Bromelin	0.076	0.453
Ficin	0.108	0.175
Pepsin	1.558	0.436
Trypsin	0.159	0.481
Control	1.894	0.494

trated papain. This is an exaggerated effect caused by the dilution of papain in the commercial meat tenderizer.

The three plant enzymes had approximately the same proteolytic activity (hemoglobin assay) and are similar in their basic properties and mechanisms of action. There were no pronounced differences between these three enzymes.

Of the three fungal enzymes, Rhozyme P-11 is the superior proteolytic enzyme on a weight basis (hemoglobin assay) and gave better hydrolysis of muscle proteins.

Because of the relatively high pH (5.5) of the beef substrate compared with the optimum pH of pepsin, it is apparent from these results that pepsin cannot be applied as a meat tenderizer. In contrast, trypsin showed a large relative hydrolysis of muscle proteins.

Both collagen and elastin, which together comprise the connective tissue of meat, the fraction to which toughness has often been attributed, were found to be digested by one or more of the enzymes. Papain and ficin were found to digest elastin, while Rhozyme P-11, Protease 15, and Rhozyme A-4 show slight but definite digestion of elastin.

In the case of collagen the greatest digestion was found with bromelin, ficin, and trypsin, followed by Rhozyme P-11 and papain. The apparent digestion of collagen may possibly result from the exaggerated circumstances of digestion, i.e., digestion for one hour at 60°C. However, since no reduction of collagen occurred during the incubation of control samples for one hour at 60°C., apparently the relatively high digestion temperature was not solely responsible for this reduction. Other investigations of papain conducted in this laboratory gave evidence that tendon collagen, prepared according to the method of Baker, Lampitt, and Brown *(2)*, was slowly

TABLE 5

Relative hydrolysis of muscle proteins by various proteolytic enzymes.

Source of enzyme	Enzyme	Relative hydrolysis of muscle proteins
Plant, papaya	Papain	1.00
Plant, pineapple	Bromelin	1.02
Plant, fig	Ficin	0.85
Plant, papaya	Commercial meat tenderizer	28
Fungi	Rhozyme P-11	1.29
Fungi	Rhozyme A-4	0.80
Fungi	Protease 15	1.11
Animal, gastric mucosa	Pepsin	0.015
Animal, pancreas	Trypsin	16

hydrolyzed by purified papain prepared according to the method of Kimmel and Smith *(9)*. This finding corroborates previous evidence presented by Sizer for the slow digestion of collagen by papain *(18)*.

An over-all evaluation of the results indicates that bromelin, ficin, trypsin, papain, and Rhozyme P-11 are all powerful proteolytic enzymes which deserve further attention as enzymes for meat tenderizers.

A more definite recommendation for the use of one of these five enzymes over the others cannot be made on the basis of this research alone. There is need for further experimental information on enzymic tenderization of whole steaks, etc., using mechanical and organoleptic evaluation. Also, final choice of one enzyme must involve due consideration of the advantages of low cost, good storage stability, high inactivation temperature for maximum effectiveness during cooking, absence of unusual odors, and proven absence of toxic effects.

SUMMARY

For extraction of meat proteins 0.1 N sodium hydroxide proved superior to 0.5% potassium chloride or 20% urea. The collagen and elastin determinations based on sodium hydroxide extraction were in good agreement with the hydroxyproline determinations.

Papain, bromelin, ficin, trypsin, and Rhozyme P-11 gave good hydrolysis of soluble beef proteins. Papain and ficin were found to hydrolyze elastin, and bromelin, ficin, trypsin, papain, and Rhozyme P-11 were found to hydrolyze collagen.

On the basis of this research, papain, bromelin, ficin, trypsin, and Rhozyme P-11 appear to have the necessary proteolytic activity for use in meat tenderizers.

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EFFECT IN SURFACE RIPENED CHEESE OF IRRADIATION ON SPORES AND TOXIN OF *CLOSTRIDIUM BOTULINUM* TYPES A AND B

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Cheese spreads are not sterile; consequently, any heat-resistant organisms (spores) that survive pasteurization can grow if the medium is not inhibitory. Milk, the basic ingredient in the manufacture of cheese, contains saprophytic spore-forming anaerobic bacteria. These organisms, including *Clostridium botulinum* whose natural habitat is soil, can sometimes gain access to cheese during the processing and curing operations. Heat treatment sufficient to produce a sterile product causes undesirable flavor changes, and has not proved commercially feasible. An alternative method of sterilizing cheese spreads in the consumer package is desirable. Radiation as a method of inactivating spoilage organisms was investigated. *Cl. botulinum* spores are heat resistant and must be considered in connection with vacuum packed cheese products.

One phase of these studies was concerned with the effect of irradiation with Cobalt-60 on the spores of *Cl. botulinum*, types A and B, when they were suspended in a soft surface ripened cheese medium. The irradiation dosage above which spores of *Cl. botulinum* cease to grow and produce toxin in the irradiated cheese was determined by conducting several trials with toxin-free spores of each of four strains of *Cl. botulinum* (3 type A and 1 type B). Also, an attempt was made to estimate the number of viable spores remaining in surface ripened cheese after varying dosages of irradiation with Cobalt-60.

Studies were also conducted on the effect of gamma irradiation on *Cl. botulinum* toxin in both broth and surface ripened cheese mediums. The extremely high dosages of irradiation required to inactivate these toxic preparations was somewhat unexpected. These experiments were designed to determine: (a) the irradiation dosage required to reduce the toxicity of cheese or broth samples containing varying initial toxin levels to the minimum level measurable by intraperitoneal mouse injections (in this instance to less than 20 m.l.d. per gram of sample), and (b) the general pattern of reduction in toxicity as progressively higher doses of irradiation were applied to similar toxic samples.

MATERIALS AND METHODS

In the experiments in which spores were to be irradiated, moderately ripened Liederkranz cheese diluted with approximately 10% distilled water was warmed in a water bath at 70°C. and stirred until homogeneous. A sufficient quantity of well-mixed cheese was accurately weighed into a beaker; then, the cheese was heated in a water bath at 90°C. for 10 minutes. Immediately after pasteurization the cheese was cooled to below 50°C. Sterile distilled water was then added to restore the moisture lost during heating. After inoculating the cheese with 10,000 toxin-free spores per gram, 6-g. portions were weighed into sterile 15 × 125 mm. culture tubes. The section of the tube approximately one inch above the top level of the cheese was constricted; then,

the air from above the cheese was evacuated and replaced with a mixture of 90% N_2 and 10% CO_2. The final vacuum level in the sealed tubes was 400 mm. Hg.

Six sealed tubes were irradiated at each exposure. Analyses for *Cl. botulinum* toxin were conducted on samples inoculated with type A spores after 3, 7, 14, 30, and 60 days of incubation at 30°C. (with the exception of Experiment 4 in which samples were not analyzed at 7 or 30 days); with type B the analyses were at 7, 14, 30, 60, and 90 days. At each analysis an unirradiated control sample was tested parellel with the irradiated specimen. In addition, an uninoculated, unirradiated sample was tested with each series. Toxin was assayed by intraperitoneal injection of white mice with 0.3 ml. portions of the supernatant fluid from centrifuged 1 to 5 aqueous dilutions of the cheese.

In the experiments in which broth containing *Cl. botulinum* toxin was added to cheese, Liederkranz cheese was warmed in a water bath at 70°C. and stirred until homogeneous. The cheese was weighed into a beaker, pasteurized, and cooled to below 50°C. Sterile distilled water was added to restore the moisture lost during pasteurization. Sufficient Seitz-filtered toxic broth (usually 10 to 15%) was added to yield the desired toxin level in the cheese. Four-gram portions were weighed into sterile 15 × 125 mm. culture tubes. The air above the cheese was partially evacuated before sealing. In some experiments, the air was replaced with a mixture of 90% N_2 and 10% CO_2.

The toxin in the cheese and broth samples was assayed at 3 levels; namely, 1000, 200, and 20 m.l.d. The lowest level that could be detected in cheese was 20 m.l.d. without encountering non-specific deaths from breakdown products. To keep the data comparable, similar titration levels were used for the broth samples.

In several of the experiments, the approximate number of spores remaining in experimentally inoculated surface ripened cheese after varying dosages of irradiation was determined. In 4 tests, spore counts were made in tubes of beef infusion agar. The resultant data gave the approximate residual viable spore level, although colony counts of dilutions of cheese are difficult to interpret because of the turbidity and small particles contributed by the cheese which may have the appearance of colonies. In another experiment the numbers of viable spores remaining in irradiated and control samples were determined by the dilution extinction technique with tubes of beef heart broth used as the recovery medium. Four dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were inoculated with cheese at each analysis (initially and after 3, 14, and 60 days of incubation at 30°C.). The inoculated beef heart tubes were incubated for 10 days at 30°C. before white mice were injected intraperitoneally with 0.5 ml. portions of culture from each tube. The presence of botulinum toxin showed the highest dilution containing viable *Cl. botulinum* spores. All positive samples were rechecked with types A and B antitoxins.

RESULTS

Effect of irradiated spores of Clostridium botulinum in surface ripened cheese. In Table 1 data from 8 experiments on soft surface ripened cheese samples inoculated with *Cl. botulinum* spores and irradiated at various dosages of gamma irradiation with Cobalt-60 are shown. The irradiation levels that inhibited growth and toxin production by each of 3 type A strains and 1 type B strain are presented in Table 1. Data furnished by the Health Physics Department of the University of Chicago were used in calculating the irradiation dosages.

Table 1 shows that there was reasonably good agreement in the levels of gamma irradiation tolerated by spores of each strain of *Cl. botulinum* when tested in separate experiments. In general, spores of type A survived 800,000 and were inactivated by 1,100,000 rep.

The spores of one type B strain of *Cl. botulinum* (126.7B) were less resistant than type A spores when irradiated in cheese. Spores of culture 126.7B survived 400,000 but were inactivated by 540,000 rep.

The results on the three type A strains of *Cl. botulinum* showed that incubation beyond 7 days at 30°C. rarely increased the irradiation level at which toxin was produced, and this occurred only with spores of culture 109A. This observation indicated further that irradiated type A spores incapable of initiating growth in the cheese within the first week of incubation might not grow and produce toxin. No such clear-cut indications were given by the type B spores tested.

TABLE 1

Toxin production in Liederkranz cheese experimentally inoculated with toxin-free spores of Clostridium botulinum and after irradiation with Cobalt-60.[a] (Inoculum—10,000 spores per gram)

Experiment	Culture used	Dosage (rep.) (000 omitted)	Days of incubation at 30°C.				
			3	7	14	30	60
2	T (Type A)	410	D	D	D	D	D
		540	L	D	D	D	D
		810	L	D	D	D	D
		1,100	L	L	L	L	L
4	T	350	D		D		D
		520	D		D		D
		700	D		D		D
		870	L		D		D
		1,000	L		L		L
8	62 A (Type A)	540	D	D	D	D	D
		810	L	D	D	D	D
		1,100	L	L	L	L	L
		1,300	L	L	L	L	L
9	62 A	540	D	D	D	D	D
		800	L	D	D	D	D
		1,100	L	L	L	L	L
		1,300	L	L	L	L	L
11	109 A (Type A)	410	D	D	D	D	D
		550	D	D	D	D	D
		820	L	L	D	D	L
		1,100	L	L	L	L	L
13	109 A	400	D	D	D	D	D
		530	D	D	D	D	D
		790	L	L	L	L	L
		1,100	L	L	L	L	L
			7	14	30	60	90
14	126.7 B (Type B)	0	D	D	D	D	D
		410	L	D	L	L	D
		830	L	L	L	L	L
		1,700	L	L	L	L	L
16	126.7 B	0	L	L	D	D	D
		280	L	L	D	D	D
		400	L	L	L	D	D
		540	L	L	L	L	L
		800	L	L	L	L	L

L = Lived. D = Died.
[a] The temperature of irradiation varied between 3.5° and 8.0°C.

Toxin production by *Cl. botulinum* spores was inhibited in Liederkranz cheese at lower levels of gamma irradiation than had been previously reported for similar spores irradiated in other mediums *(1, 2)*. There is a possibility that products toxic to *Cl. botulinum* were formed in the cheese which inhibited growth and toxin production of spores still surviving a particular level of irradiation. Another possible explanation may be due to damaging other heat resistant organisms by irradiation which affect the growth and toxin production of *Cl. botulinum*. From the standpoint of safety an irradiation level above the highest one which permitted growth must be used in any recommendation of a sterilization irradiation dosage for cheese.

In an attempt to estimate what percentage of the inoculated spores survived varying levels of irradiation, spore counts from tubes of cheese exposed to irradiation dosages near the highest level which permitted toxin production were made. Data are shown in Table 2.

Spore counts varying from 140 to 1,500 per gram were still present in cheese samples exposed to irradiation dosages near the highest level which permitted consistent toxin production in the experiments for which the information was recorded. In Experiment 2 toxin was produced in samples irradiated at one level higher (810,000 rep) than was checked by spore recovery counts. There was close agreement in the survival counts between each pair of samples irradiated at similar dosage levels. The toxin production data in Table 1, together with the data in Table 2, showed that culture 62A was more resistant to irradiation with Cobalt-60 than either of the other two type A cultures tested.

TABLE 2

Type A Clostridium botulinum spores in Liederkranz cheese surviving varying levels of irradiation with Cobalt-60. (Initial inoculum—10,000 spores per gram)

Experiment	Culture used	Dosage (rep.) (000 omitted)	Survivors (spores per gram)	Per cent survival	Toxin produced in cheese
2	T	410	4,400	44.0	Yes
		540	1,500	15.0	Yes
8	62 A	810	140	1.4	Yes
		1,100	<10	<0.1	No
9	62 A	800	300	3.0	Yes
		1,100	<10	<0.1	No
13	109 A	400	Counts unsatisfactory		Yes
		530	700	7.0	Yes

Results from experiments designed to study the effect of irradiation on the toxin producing ability of spores showed that this characteristic was unaltered by sub-lethal dosages. Spores that survived a dosage of irradiation slightly below that which inhibited growth and toxin production when the irradiated cheese was incubated at 30°C. yielded colonies in agar that produced *Cl. botulinum* toxin in the beef heart broth of a potency equal to the titre of similar toxin from non-irradiated *Cl. botulinum* spores.

In two experiments the number of viable spores remaining in experimentally inoculated Liederkranz cheese after being subjected to varying dosages of gamma irradiation was estimated by the dilution extinction technique using beef heart broth as the recovery medium. Spore counts on these samples are shown in Table 3. The number of spores recovered from the unirradiated controls or after an irradiation dosage of 350,000 rep was consistently near the estimated number inoculated (10,000 per gram). At progressively higher dosages the number recovered initially (no incubation of cheese at 30°C.) dropped steadily until the samples had received 1,000,000 rep, after which all beef heart broth tubes of the 1:10 dilution were negative (less than 10 viable spores per gram). All spores were not inactivated by the highest irradiation level tested (1,400,000 rep), as indicated by the number of spores recovered after incubation of the irradiated cheese samples for 3, 14, or 60 days at 30°C. prior to analysis. The greater number of spores observed at some of these later analyses are, of course, mainly from subsequent generations of vegetative cells that ceased active growth and returned to the spore state. Apparently the amount of growth and reproduction that took place in these samples of irradiated cheese was not very extensive because in no case was toxin of detectable concentration formed after the initial spore count had been reduced to below 10 spores per gram. Toxin was produced in Experiment 4 in samples that received an irradiation dosage of 870,000 rep, but not when the inoculated cheese was subjected to 1,000,000 rep. The critical irradiation level was lower in Experiment 5. In this instance 860,000 rep was sufficient to stop toxin

TABLE 3

Type A Clostridium botulinum spores recovered from Liederkranz cheese after varying levels of irradiation with Cobalt-60.[a] (Initial inoculum—10,000 spores per gram)

Expt.	Culture used	Dosage (rep.)	Number of spores per gram recovered from cheese incubated at 30°C. for the following number of days before analyses				Toxin produced in cheese
			0	3	14	60	
4	T	None	1,000	10,000	10,000	10,000	Yes
		350,000	1,000	1,000	10,000	1,000	Yes
		520,000	100	100	1,000	1,000	Yes
		700,000	100	100	10,000	10,000	Yes
		870,000	100	100	100	<10	Yes
		1,000,000	<10	<10	<10	<10	No
		1,400,000	<10	<10	10	<10	No
5	T	None	10,000	10,000	10,000	10,000	Yes
		340,000	10,000	10,000	1,000	100	Yes
		520,000	1,000	1,000	1,000	1,000	Yes
		690,000	100	10	10,000	<10	Yes
		860,000	100	<10	100	10	No
		1,000,000	<10	1,000	10	10	No
		1,400,000	<10	10	1,000	<10	No

[a] The inoculated cheese samples were analyzed in duplicate for spores and toxin. Uninoculated control cheese samples were analyzed singly at three of the six trials in each experiment.

production in the irradiated cheese. The fact that toxin was not formed in irradiated Liederkranz cheese at a dosage level well below that needed to kill all the *Cl. botulinum* spores indicated that: (a) some changes had taken place in the cheese which made it a poorer medium than the unirradiated control sample or (b) irradiation may selectively remove certain microorganisms allowing others to grow which interfere with *Cl. botulinum* activity. Furthermore, the difference in the activity of similar levels of viable spores in two different lots of cheese subjected to irradiation with 870,000 rep indicated that either there were varying levels of inhibitory substances generated in the same kind of cheese by this dosage of gamma irradiation or the spores used in Experiment 4 were more active than those used in Experiment 5.

EFFECT OF IRRADIATING TOXIN OF CLOSTRIDIUM BOTULINUM IN SURFACE RIPENED CHEESE

Preliminary experiments designed to test the effect of gamma irradiation of *Cl. botulinum* toxin inoculated into surface ripened cheese showed that levels of irradiation much higher than those anticipated were required to inactivate botulinum toxin in this medium. Therefore, large irradiation dosages were used on Liederkranz cheese inoculated with type A *Cl. botulinum* toxin produced in trypticase broth (see Table 4). In one trial with toxin produced by Culture T, the dosage required to reduce the toxicity from above 1,000 m.l.d. to below 20 m.l.d. was 7,800,000 rep. Similar results were observed in three trials with toxin produced by Culture 109A. In Trial 1 the toxicity was not reduced from above 1,000 m.l.d. to below 20 m.l.d. by 7,800,000 rep. In Trial 2 this dosage was sufficient to reduce a comparable level of initial toxicity to less than 20 m.l.d. In Trial 3 a toxicity above 1,000 m.l.d. was not reduced to below 20 m.l.d. by 7,200,000 rep. These levels of irradiation were much higher than those required to inactivate *Cl. botulinum* spores in surface ripened cheese, and slightly higher than the dosage required to reduce similar levels of type A *Cl. botulinum* toxin in broth to a comparable end point. In the interpretation

TABLE 4

The effect of varying dosages of irradiation with Cobalt-60 on Clostridium botulinum toxin in Liederkranz cheese in partially filled sealed tubes. (Atmosphere above cheese in tubes was 90% N_2 + 10% CO_2)

Irradiation dosage (rep.) (000 omitted)	Toxin produced by			
	Culture T (type A) Trial 1 m.l.d. toxin	Culture 109A (type A)		
		Trial 1 m.l.d. toxin	Trial 2 m.l.d. toxin	Trial 3 m.l.d. toxin
None	1,000[a]	1,000	1,000	1,000
350	1,000	1,000	1,000	1,000
530	1,000	1,000	1,000	1,000
700	200[b]	1,000	1,000	1,000
860				1,000
1,000	200	1,000	1,000	200
1,400	200	200	200	200
1,700	200	200	200	200
2,100	200	200	200	200
3,100	200	200	200	20
4,200	20[c]	200	200	20
5,200	20	20	20	20
6,300	20	20	20	20
7,200				20
7,800	<20[d]	20	<20	

[a] Between 1,000 and 10,000 m.l.d.
[b] Between 200 and 1,000 m.l.d.
[c] Between 20 and 200 m.l.d.
[d] Less than 20 m.l.d.

of the data in Table 4 it should be pointed out that toxin was assayed for by duplicate intraperitoneal injections into mice at each of three levels; namely, 1,000, 200, and 20 m.l.d. Therefore, in the tables, 1,000 m.l.d. indicates 1,000 m.l.d. or more (but below 10,000 m.l.d.), 200 m.l.d. indicates between 200 and 1,000 m.l.d. and 20 m.l.d. indicates between 20 and 200 m.l.d.

EFFECT OF IRRADIATING TOXIN OF CLOSTRIDIUM BOTULINUM IN TRYPTICASE BROTH

After observing the slow inactivation of *Cl. botulinum* toxin in cheese by irradiation with Cobalt-60, it was suspected that this medium possibly contributed protection to the toxin. Therefore, several experiments were conducted in which *Cl. botulinum* in trypticase broth was irradiated at varying dosages.

The data on some of the earlier trials with trypticase broth containing *Cl. botulinum* did not extend to irradiation dosages high enough to include the reduction of the toxicity of the test samples to below 20 m.l.d. This level was chosen as the lowest test point so that the results would be comparable to similar data on *Cl. botulinum* toxin added to cheese.

In Table 5 four experiments with toxic trypticase broths having a low initial toxicity are presented. For these experiments the toxin was produced by culture T. The atmosphere above the broth in the partially filled sealed tubes was varied to test its effect on the susceptibility of the crude toxin to gamma irradiation.

An examination of Table 5 showed that there was no apparent differential influence of any of these atmospheres on the rate of inactivation of

TABLE 5

The effect of varying the atmosphere above toxic trypticase broth in partially filled sealed tubes on the inactivation of Clostridium botulinum toxin irradiated with Cobalt-60. (Toxin produced by Culture T, Type A)

Irradiation dosage (rep.) (000 omitted)	Atmosphere above broth in tube			
	CO_2	90% N_2 + 10% CO_2	Air (Trial 1)	Air (Trial 2)
	m.l.d. toxin	m.l.d. toxin	m.l.d. toxin	m.l.d. toxin
None	20[a]	20	20	20
370	20	20	20	20
560	20	20	20	20
740	20	20	20	20
1,100	20	20	20	20
1,500	20	20	20	20
1,800		20	20	20
2,200		20	20	<20
3,300				<20
3,800				<20
4,400		<20[b]		

[a] Between 20 and 200 m.l.d.
[b] Less than 20 m.l.d.

the toxin in the broth. Further, with broth containing between 20 and 200 m.l.d. of *Cl. botulinum* toxin, at least 2,200,000 rep were required to reduce the toxicity to below 20 m.l.d. Insufficient samples were available of some of the experimental series to reach the proposed end point; hence, specific dosages required for inactivation were not established in all trials. The large dosage of gamma irradiation required to inactivate *Cl. botulinum* toxin was in itself somewhat unexpected, because of its heat lability. Similar results were obtained when trypticase broth containing toxin produced by culture 109A (type A) was irradiated in sealed tubes having one of the three atmospheres tested above in the headspace over the broth.

In an attempt to obtain broth cultures with a higher initial toxicity, cultures T and 109A were grown in beef heart broth with meat present. Data from four experiments with toxic beef heart broths are in Table 6.

According to the data in Table 6 the general pattern of inactivation of the toxin was the same in these experiments as with the toxic trypticase broths. The higher initial toxicity of the beef heart broth samples irradiated required higher irradiation levels for the reduction of the toxin to 20 m.l.d. (or less than 20 m.l.d. on one trial).

The experimental data with broths containing *Cl. botulinum* toxin showed that there was an orderly destruction of the toxicity during the extended irradiation exposure required to reduce the varying initial amounts of toxin to the proposed endpoint (less than 20 m.l.d.). In the experiments where the initial toxin level was somewhat above 20 m.l.d., reduction of the toxicity to the end point required at least 2,200,000 rep. When the initial toxicity was somewhat above 200 m.l.d., reduction to below 20 m.l.d. required at least 4,200,000 rep. Toxin levels above 1,000 m.l.d. were not reduced to below 20 m.l.d. by less than 4,300,000 rep, and in most instances by at least 5,300,000 rep.

A single experiment with toxic broths from each of cultures 62A (type A) and 126.7B (type B) was conducted. The initial toxin levels were

TABLE 6

The effect of varying dosages of irradiation with Cobalt-60 on Type A Clostridium botulinum toxin in beef heart broth in partially filled sealed tubes (Atmosphere over broth in tubes was air)

Irradiation dosage (rep.) (000 omitted)	Toxin produced by			
	Culture T		Culture 109A	
	Trial 1	Trial 2	Trial 1	Trial 2
	m.l.d. toxin	m.l.d. toxin	m.l.d. toxin	m.l.d. toxin
None	1,000[a]	1,000	1,000	1,000
360	1,000	200	1,000	1,000
540	200[b]	200	1,000	1,000
720	200	200	1,000	1,000
1,100	200	200	200	1,000
1,400	200	200	200	1,000
1,800	200	200	200	1,000
2,200	200	20	200	1,000
2,700	20[c]		200	
3,200	20	20	20	200
3,800	20		20	
4,300	20	<20[d]	20	20
5,300		<20		20

[a] Between 1,000 and 10,000 m.l.d.
[b] Between 200 and 1,000 m.l.d.
[c] Between 20 and 200 m.l.d.
[d] Less than 20 m.l.d.

somewhere between 200 and 1,000 m.l.d. An inactivation pattern was observed similar to that with toxic broths from the other two type A cultures. From the data presented one cannot predict accurately whether toxin from any one of the *Cl. botulinum* strains tested was more resistant to irradiation than the others.

Two experiments were conducted in which the pH levels of toxic trypticase broths were adjusted downward from approximately 7.5 to near pH 5.5 with HCl. The general pattern of inactivation of the toxin appeared to be very similar to that for broths at pH levels nearer neutrality, except that the rate of inactivation seemed to increase somewhat at the higher irradiation dosage levels. Additional trials would be needed to establish this observation conclusively. The dosage required to reduce the toxicity to below 20 m.l.d. was 4,200,000 rep when the initial level was between 200 and 1,000 m.l.d. and 5,300,000 rep when the initial level was above 1,000 m.l.d.

THE EFFECT UPON THE TITRE OF BOTULINUM TOXIN MIXED IN IRRADIATED CHEESE AND BROTH AND STORED AT 3°C.

There was a possibility that some of the effects of irradiation with Cobalt-60 would be transient and reversible. To determine whether samples irradiated with varying dosages of gamma irradiation maintained their initial toxicity, a duplicate set of samples from each experiment was analyzed 2 weeks after the initial analyses. In a few instances there was an apparent increase in toxicity, although more often there was a decrease in the toxin level. However, approximately 77% of the samples had the same toxin level initially and after 2 weeks of storage at 3°C. An analysis

of comparable data collected on unirradiated control samples gave similar results. Among these samples 74% had the same toxin level initially and after 2 weeks of storage.

SUMMARY

Data are presented from 8 experiments on soft surface ripened cheese samples inoculated with *Clostridium botulinum* spores and irradiated at various dosages with Cobalt-60. There were some variations in the levels of gamma irradiation tolerated by spores of the same strain of *Cl. botulinum* when tested in separate experiments. It was shown that spores of culture 62A were more resistant to irradiation than the spores of either of the other type A strains tested. The spores of one type B strain were inactivated by lower dosages of irradiation than were type A spores. In general, type A spores were inactivated by 800,000 to 1,100,000 rep; type B spores by 550,000 rep. Results from experiments initiated to study the effect of irradiation with Cobalt-60 on the toxin producing ability of spores reflected that this characteristic was unaltered by sub-lethal dosages.

Preliminary experiments designed to test the effect of gamma irradiation on *Cl. botulinum* toxin inoculated into surface ripened cheese disclosed that levels of irradiation higher than anticipated were required to inactivate botulinum toxin in this medium. Similar results were observed when crude toxin preparations grown in either trypticase or beef heart broth were irradiated with Cobalt-60. In the trials with broth or cheese containing *Cl. botulinum* toxin, the toxicity was assayed at three levels; namely, 1,000, 200, and 20 m.l.d. Insufficient samples of some of the experimental series were available to reach the proposed end point (less than 20 m.l.d.); hence, specified dosages required for inactivation were not established in all trials.

Type A *Cl. botulinum* toxin added to cheese required higher levels of irradiation to reduce the toxicity to below 20 m.l.d. than was observed with toxin irradiated in a broth medium. In cheese, where the initial toxin level was above 1,000 m.l.d., reduction of the toxicity to below 20 m.l.d. required at least 7,800,000 rep.

There was no apparent differential influence on the rate of inactivation of the toxin in trypticase broth, whether the atmosphere above the broth in the sealed tubes was CO_2, a mixture of 90% N_2 and 10% CO_2, or air. Again, the pattern of inactivation for toxin in beef heart broth appeared similar for the toxin in trypticase broth. In broth, where the initial toxin level was somewhat above 20 m.l.d., reduction of the toxicity to the end point (less than 20 m.l.d.) required at least 2,200,000 rep. When the initial toxicity was above 200 m.l.d. reduction to below 20 m.l.d. required at least 4,200,000 rep. Toxin levels above 1,000 m.l.d. were not reduced to below 20 m.l.d. by less than 4,300,000 rep, and in most instances at least 5,300,000 rep were required.

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A CRITICAL COMPARISON OF THE TWO-SAMPLE AND TRIANGULAR BINOMIAL DESIGNS

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The relative sensitivities of the two-sample and triangular designs, in cases where quality may be stated unambiguously, have been much discussed. From a classical standpoint, using a binomial mathematical model, the triangular technique should be more efficient than the two-sample *(9)*. This arises from the differences in the chance probability for each design.

The two-sample comparison consists in the presentation of two samples (different in intensity of quality) to the taster, who is required to denote one as being higher in intensity. The chance probability for this occurrence to be correct is one-half. The triangular test comprises three samples, two identical and one different in intensity of taste quality. The taster is required to determine the odd sample. By chance, this probability is one-third.

When the tests are given in the above manner, actual experimental results have shown that the triangular design is not as sensitive as the two-sample method in the determination of thresholds *(2, 4)*. This phenomenon has been explained as being due, to a large extent, to the inherent limitations of flavor perception *(2)*. However, this view is not unanimous. Protracted tasting is not necessarily the cause for fatigue or sensory adaptation *(11)*. An attempt has been made to increase the efficiency of the triangular design by including the partially correct judgments in tests of significance *(3)*.

Using these designs, the levels of significance for thresholds have been established by employing only the probability of committing an error of the first kind; i.e., the chance that the hypothesis of no real difference may be rejected when it is true. However, it has been emphasized that a consideration of the error of the second kind (the chance that a taster may overlook a real difference when it exists) is of at least as great importance as that of the error of the first kind *(1)*.

It is the purpose of this paper to demonstrate, by experiment, inherent differences existing between the two-sample and the triangular designs. A consideration of the error of the second kind will be used to establish two defined types of statistically significant threshold, (a) the absolute threshold, defined as the minimum detectable concentration and (b) the difference threshold, the minimum detectable concentration difference.

Theoretical considerations. Fechner's law has been stated as follows *(5)*:

$$S = A\log R + B \qquad \text{(a)}$$

where S is the sensation produced by the stimulus R; A is a constant depending upon the quality being tested and upon the sensory characteristics and B is a constant expressing a unit of just noticeable difference.

It has been suggested that the percentage of correct judgments, corrected for chance, may be used as a measure of sensation *(7)*. The following equation has been derived *(5, 8)*:

$$P = \frac{100\,(T - E)}{100 - E} \qquad \text{(b)}$$

where P = % correct judgments corrected for chance expectation,
T = % observed correct judgments,
E = % correct judgments expected by chance.

If the concentration difference, $C - C_o$, may be assumed to measure the stimulus R, and P to replace the resulting sensation, S, equation (a) takes the following form:

$$P = A' \log\,(C - C_o) + B' \qquad \text{(c)}$$

where A′ and B′ are constants similar to A and B in equation (a).

In order to find the value of B′, the following substitutions may be made: Let $C' - C_o$ be the value of $C - C_o$ when $P = 0$, $C' - C_o$ being the minimum difference capable of causing sensation. It is obvious that any smaller difference would be meaningless. Therefore,

$$B' = -A' \log\,(C - C_o)$$

The complete equation then becomes

$$P = A' \log\,(C - C_o) - A' \log\,(C' - C_o)$$

$$= A' \log \frac{C - C_o}{C' - C_o} \qquad \text{(d)}$$

The difference $C' - C_o$ will be designated as the just noticeable difference (j.n.d.), which is of little statistical value, to differentiate from the absolute and difference thresholds defined in this paper as statistically significant limens.

P is a monotonic function, being zero below the point of a j.n.d., increasing with increasing differences to 100% at a point where the difference is always detectable, beyond which P remains constant. This range for P (0-100%) may be regarded as a twilight region, a region of uncertainty, in which part of the time a response occurs and part of the time it does not *(5)*. For a single stimulus, it is plausible that P is linearly related to the error of the second kind. A logical stimulus threshold would be one for the value at P = 50%, since there would be a probability of 0.50 for producing a response at this point.

EXPERIMENTAL

Aqueous solutions of sucrose were used for the tests. Several concentrations were used in each series in order to obtain from four to seven points in the psychological continuum, P, from 0% to 100%, as calculated from equation (b).

One series comprised distilled water compared with sucrose solutions varying in concentration from 0.025% to 0.450%. In another series, a 1% sucrose solution was compared with solutions ranging from 1.025% to 1.350%. Similarly, a 10% solution was compared with solutions in the range of 10.05% to 10.80%.

In general, the tests were given to one taster only. The comparisons were presented alternately in triangular (for determination of odd sample) and two-sample (for judgment of sample of higher intensity) designs. All arrangements of the designs were randomly presented. Each sitting of the taster comprised 12 sets of any one design. There were 3 to 4 sittings daily, 36 to 48 sets in all, with 5 to 10 minutes intermission

between sittings. No decrease in sensitivity was found on repetitive tasting, an observation noted in other investigations *(11)*. The taster preferred not to rinse orally between samples.

From 60 to 90 separate judgments were made for each concentration comparison in each series for each design. This was done to obtain a fairly stable mean value for P, the percentage of correct judgments over chance, for each comparison in the psychological continuum.

For each series all of the concentration comparisons were randomized in presentation to avoid the effect, noted in a previous work *(6)*, that the number of correct judgments plotted against the concentration produced two different curves depending upon whether the samples had been presented in order of increasing or decreasing concentration.

The values for P were plotted as abscissas against the logarithms of the concentration differences, ΔC, as ordinates for each design in each series. Assuming a linear relationship and using the method of least squares, lines of best fit were drawn connecting the points.

A twelve-member panel was used for some of the tests to substantiate results obtained by the single taster.

RESULTS AND DISCUSSION

Figure 1 shows, for the one taster, the comparison of the triangular and two-sample designs for sucrose solutions tested against distilled water. There is a sharp break in response for the triangular at the 0.10% sucrose

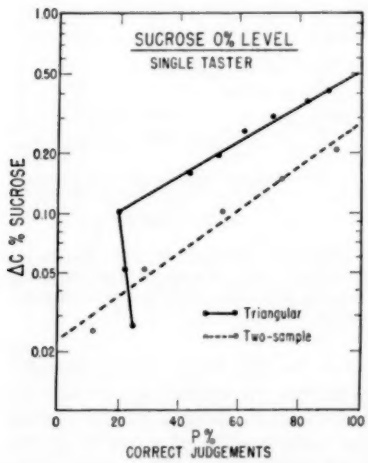

Figure 1. Comparison of response for triangular and two-sample designs, using sucrose at 0% level and a single taster.

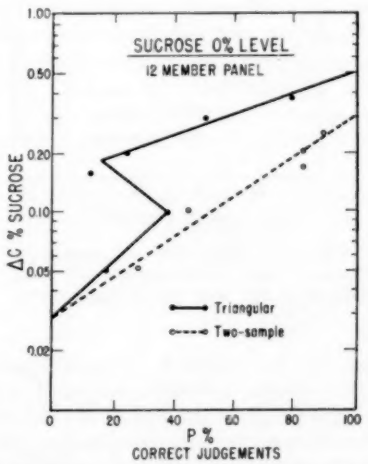

Figure 2. Comparison of response for triangular and two-sample designs, using sucrose at 0% level and a 12-member panel.

concentration. Therefore, the fitted line was calculated for the higher concentrations only.

To demonstrate that the above effect was not unique to the single taster, a similar test was given to a panel of 12 members, a total of 50 judgments being taken for each concentration comparison. Figure 2 reveals the same lag in response for the triangular test.

The sharp change in response was very definite and not due to chance causes. The significance of the lag is not clear. Possibly, at the lower concentration, the taster does not recognize the taste quality and the inflection point represents the transition from non-cognizance to recognition. It has been reported that although some judges could not actually name a taste until a relatively high concentration was reached, they could often tell that there were different concentrations of this unnamed substance even though they were below their threshold *(10)*.

Further evidence for the theory that the taster may not be able to recognize the taste quality at lower concentrations is found in Figures 3

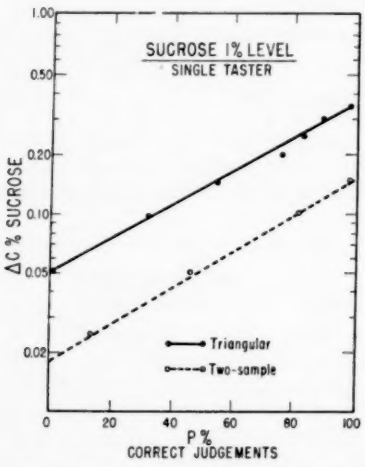

Figure 3. Comparison of response for triangular and two-sample designs, using sucrose at 1% level and a single taster.

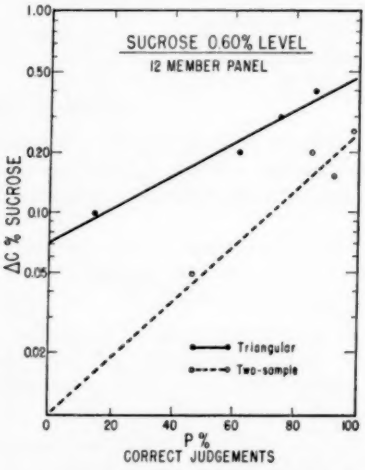

Figure 4. Comparison of response for triangular and two-sample designs, using sucrose at 0.60% level and a 12-member panel.

and 4. In Figure 3, the results are plotted for the single taster, using a 1% sucrose solution compared with solutions of higher concentration. Figure 4 plots the results for the 12 member panel, using a 0.60% sucrose solution as the base comparison. Since the taste quality of a 0.60% or 1% sucrose solution is easily recognizable by all tasters, the only decision to be made is a matter of relative intensity. The lag in response has disappeared.

The important note, however, is the fact that the two-sample design as given is more sensitive than the triangular for the entire range of the continuum. Figure 5 further demonstrates the difference between the two designs, in this case at a sugar concentration level of 10% for the single taster.

The degree of linearity for the fitted lines in Figures 1 to 5 was tested by calculation of the product-moment correlation coefficient for each line. The values for the correlation coefficients, given in Table 1, demonstrate a linear relationship, to a highly significant degree, between log Δ C and P.

TABLE 1

Product-moment correlation coefficients for fitted lines of Figures 1-5.

Figure	Concentration level	Triangular	Two-sample
1	0% single taster	0.99	0.98
2	0% 12-member panel	0.98	0.99
3	1% single taster	0.99	0.99
4	.60% 12-member panel	0.97	0.90
5	10% single taster	0.99	0.97

In these experiments it appears that Fechner's law is obeyed. Only one value, that for the two-sample at 0.60% sucrose with a 12-member panel, did not yield significance at the 5% level.

To determine the nature of the difference between the two techniques, two variations were presented to the single taster. One variation was in the two-sample design. The 1% sucrose concentration level was used and the presentation included four arrangements, HL, LH, LL, and HH, where L = 1% sucrose and H = higher than 1% sucrose. The range for H covered concentrations from 1.10% to 1.35%. The question asked was, "Are these two samples the same or different?" The other variation was a presentation of the triangular test in which the taster was apprised of the fact that the solution of higher concentration was always maintained as the odd sample. Sucrose at the 1% level was the basis of this test, also.

Figure 6 shows the results of these determinations. The lines of response for the usual triangular and two-sample designs are designated by the unbroken line (——) and by (- - - -); the variation of the two-sample, represented by the line (— —), causes a decrease in sensitivity, this

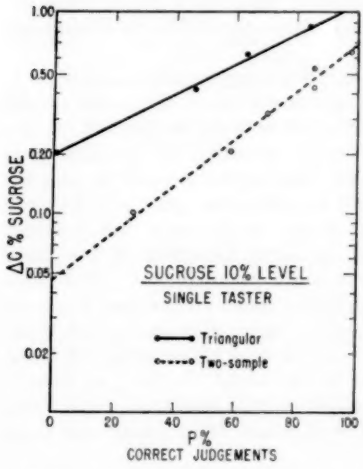

Figure 5. Comparison of response for triangular and two-sample designs, using sucrose at 10% level and a single taster.

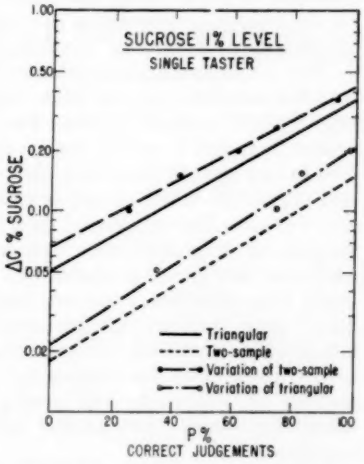

Figure 6. Comparison of response for various variations of triangular and two-sample designs, using sucrose at 1% level and a single taster.

presentation now being weaker than even the usual triangular method. This may explain the lower efficiency of the triangular test. When comparing the samples in triangular testing, it is usual to compare two samples at a time. From the nature of the test, any two compared samples will be the same or different equally often. Therefore, the judging of two samples of the three presented in the triangular technique requires the same type of decision as was used in the variation of the two-sample. The fact that the triangular is somewhat stronger than the variation of the two-sample suggests that the third sample aids in making a decision as to the quality of the other two.

The response line for the variation of the triangular test (where the higher concentration is always the odd sample), is given by the line (— . —), in Figure 6. In this case the sensitivity of the design has been increased. By earmarking the higher concentration as the odd sample, the effect has been to approach the sensitivity of the usual two-sample comparison.

The above results give an indication that there are psychological obstacles introduced when the third sample is added. It is to be expected that more complex quality systems would show even greater variance.

In general, the primary difference between the two designs lies in the just noticeable difference (j.n.d.). This is the concentration difference at $P = 0$. It is for this reason that the statistical interpretation fails when considering the binomial distribution as the mathematical model. The model assumes that the point of j.n.d. is identical for both designs and that the triangular is more discriminating due to differences in chance probabilities. Comparison of the slopes of the lines for each of Figures 2, 4, and 5, in particular, clearly demonstrates the superior power for discrimination of the triangular design. However, the greater j.n.d. necessary for the triangular overcomes any statistical advantage in the determination of absolute or difference thresholds.

For the determination of absolute and difference thresholds, disregarding characteristics of the individual, a consideration of the error of the second kind is advantageous. For example, if the intersection of the response line with $P = 50\%$ represents the point at which the probability is 0.50 for a response to a particular stimulus, this point may well be adopted as a threshold. It has the advantage that it is a definable point and does not depend upon the number of judgments, except in the way that a larger number of judgments may decrease the standard deviation of the mean value for this point or threshold. It is a stable threshold. On the other hand, any statistical inference based upon the error of the first kind, an unchanging fundamental probability, depends upon the number of judgments. The threshold value so obtained varies with the number of judgments, the greater the number the lower the threshold. In fact, an infinite number of judgments will give a threshold at the point of j.n.d. This instability causes incompatible thresholds obtained from small and large numbers.

SUMMARY

When the quality may be stated unambiguously, the two-sample design has been found more sensitive than the triangular. This difference in power

of discrimination is explained by the fact that the just noticeable difference for the triangular is higher than for the two-sample design.

The response curves are linear, plotting logarithm of the concentration difference against percentage correct judgments above chance, and Fechner's law appears to be obeyed for the sucrose solutions used.

A consideration of the error of the second kind is suggested for the determination of thresholds.

Acknowledgment

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THE CALCIUM AND PHOSPHORUS CONTENTS OF SOME FOREIGN AND DOMESTIC CANNED SARDINES[a, b]

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The minerals, calcium and phosphorus, are well established as essential for nutritional well being. Numerous vital functions have been established for these elements in addition to their function in the development of the skeletal system *(20)*. Canned fisheries products, particularly those processed with the bone included as part of the contents, have long been considered as excellent sources of these minerals.

One such product is canned sardines. In certain parts of the Orient, Asia, Africa, and South America, where dairy products are not generally available, canned sardines supply the greatest percentage of calcium and phosphorus in the diet *(19)*. In addition, this product also supplies a large percentage of the protein and the fat soluble vitamins. In the United States, sardines are considered generally as an adjunct to the diet rather than a staple. With the development of this sophistication have come several changes in preparation of the product prior to processing, such as boneless sardines, and skinless and boneless sardines.

Ichthyologically speaking, the sardine is not a particular fish, but rather the word sardine is a popular name given to certain members of a particular family of fish, the Clupeideae *(18)*. This term has been carried over into the legal definition. According to the U. S. Food and Drug Administration, a sardine is "any small, canned, clupeoid fish," and many different genera and species have been canned under the label of sardines *(14)*. There are at least four different genera falling under the general category of clupeoids. These are *Clupea*, *Sardinops*, *Sardina*, and *Alosa (18)*. In each of these genera there are several species, all of which except Alosa may be and have been canned as sardines, depending upon the location from which the can came and the species available in the local waters. In areas where two or more species abound, no attempt is made to separate the species before canning. As a result, a can of sardines may contain fish belonging to different species and, in the extreme, fish belonging to different genera. Furthermore, certain local names, such as "brisling," "sprat," and "pilchard" have been indiscriminately applied regardless of species. For example, the California sardine, *Sardinops caerula*, which is canned along the Pacific Coast from British Columbia to California, is generally known as a pilchard. However, the true pilchard *(Clupea pilchardus)* is not found in the Pacific.

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It ranges in the European Atlantic, from the English Channel southward to Portugal, and in the Mediterranean as far east as Egypt, where it is canned as a sardine *(16)*. The label usually lists the country of origin previous to the word sardine, such as Portuguese sardines, Italian sardines, or French sardines *(3, 8, 13, 14)*.

Data in the literature concerning the chemical composition of sardines record wide ranges in the calcium and phosphorus contents. Table 1

TABLE 1

Representative values of calcium and phosphorus

Description	Ca mg./100 gm.	P mg./100 gm.	Reference
Sardines	25	246	*(4)*
Sardines, canned	409	683	*(4)*
Sardines, canned in oil (drained weight)	35	365	*(1)*
Sardines, canned in oil (entire content)	29	299	*(1)*
Sardines, canned in oil	354	434	*(9)*
Sardines, canned in tomato sauce	381	168	*(9)*
Pilchards, tinned (fish only)	231	296	*(12)*
Pilchards, tinned (whole content)	190	269	*(12)*
Sardines, tinned	409	683	*(12)*
Sardines, Atlantic, in oil (entire content)	354	434	*(17)*
Sardines, Atlantic, in oil (drained weight)	386	586	*(17)*
Pilchards, Pacific, in oil (entire content)	381	168	*(17)*
Pilchards, Pacific, in tomato sauce	381	168	*(17)*

presents data from several representative references concerning the content of these minerals in canned sardines and illustrates the variations which have been recorded. Only rarely in the literature is the place of origin or the species mentioned.

Besides species differences, many other factors must be taken into consideration. There is some question as to whether or not the ash residue is constant. Dill *(6)*, who studied seasonal changes in *Sardinops caerula* and *S. japonica*, and Bruce *(5)*, who worked on *Clupea harengus*, were unable to determine any distinct seasonal changes in the amount of mineral residue in the muscular tissue. However, Lepierre's data *(10)* on the *Sardinia (Clupea pilchardus)* of the Mediterranean and the shores of Portugal show some decrease in ash when the water in the tissues is at a minimum, from August to October.

The manner in which sardines are canned is also important for the ash content of the finished product may be affected and, accordingly, the calcium and phosphorus contents.

In the first place, the question of the presence of the scales must be considered. Large fish are generally scaled before canning whereas smaller fish are generally canned without any attempt to remove the scales. Most of the calcium in fish is found in the form of a complex phosphate (apatite) or as CaCO3 in the bones, scales, otoliths, and other analogous skeletal formations. Therefore, the removal of the scales would reduce the content of calcium and phosphorus in the canned product.

Ecological conditions, such as the amount of calcium in the water and seasonal and age differences, are known to affect the calcium content. The phosphorus content in the muscles has been shown to be related to the

TABLE 2

Physical and chemical characteristics of some foreign and domestic canned sardines

Product	Sample code	Can size and net content	Oil		Av. No. fish	Av. total weight in grams	Av. drained weight in grams	Ash %	Ca mg./100 g.	P mg./100 g.	Ca:P
			Type[1]	Av. quan. in grams							
Portuguese skinless and boneless sardines	No. 12	¼ usual 22, 3¾ oz.	O	31	6	123	92	2.3	58	345	1:6
	No. 22	¼ usual 22, 3¾ oz.	O	21	5	112	91	2.1	45	340	1:7.5
	No. 86	¼ usual 22, 3¾ oz.	O	12	5	110	98	2.1	44	340	1:8
	No. 44	¼ club 30, 4½ oz.	P	27	6–5	128	101	1.8	59	305	1:5
	No. 44a	¼ club 30, 4½ oz.	O	26	6	127	101	2.0	70	325	1:5
	No. 70	¼ usual 22, 3¾ oz.	O	30	5–6	124	94	1.6	43	303	1:7
	No. 120	¼ usual 22, 3¾ oz.	O	8	5	115	107	1.6	38	314	1:8
	No. 32	¼ usual 22, 3¾ oz.	O	20	6–5	119	99	1.8	56	317	1:6
	No. 42	¼ club 30, 4½ oz.	O	28	6–5	127	99	1.7	76	282	1:4
	AVERAGE							1.9	54	320	1:6
Portuguese boneless sardines	No. 63	¼ usual 22, 3¾ oz.	O	16	6–8	113	97	3.2	493	556	1:1.1
	No. 63a	¼ usual 22, 3¾ oz.	O	13	5–10	110	97	2.9	505	558	1:1.1
	No. 42	¼ club 30, 4½ oz.	O	15	6	129	114	2.7	438	498	1:1.1
	No. 18	5¼ oz.	O	20	7	174	154	3.4	522	570	1:1.1
	AVERAGE							3.0	490	545	1:1.1
Portuguese plain sardines (whole fish)	No. 18a	¼ club 30, 4½ oz.	O	18	4	125	107	3.3	543	592	1:1.1
	No. 18b	¼ club 30, 4½ oz.	O	22	6	126	104	4.1	586	679	1:1.2
	No. 114	¼ club 30, 4½ oz.	O	26	4	125	99	4.1	475	604	1:1.3
	No. 177	¼ club 30, 4½ oz.	O	16	6	132	116	3.8	583	670	1:1.1
	No. 168	¼ club 30, 4½ oz.	O	23	10–9	130	107	3.2	510	558	1:1.1
	No. 189	¼ club 30, 4½ oz.	P	21	6	126	105	3.4	688	615	1:0.9
	No. 105	¼ club 30, 4½ oz.	P	20	6	130	110	4.1	675	654	1:1
	AVERAGE							3.7	580	624	1:1.1

Portuguese sardines in tomato sauce	No. 20	¼ club 30, 4½ oz.	O	19	10–11	129	110	4.4	431	545	1:1.3
	No. 42a	¼ club 30, 4½ oz.	P	19	6–13	129	110	3.3	480	542	1:1.1
	No. 42b	¼ club 30, 4½ oz.	O	19	8–5	129	110	3.5	664	605	1:0.9
	No. 17	¼ club 30, 4½ oz.	O	15	10	128	113	4.5	662	620	1:0.9
	No. 17a	¼ club 30, 4½ oz.	O	12	9	129	117	4.8	730	665	1:0.9
	AVERAGE							4.1	593	595	1:1
Norwegian sardines	No. 23	¼ usual 22, 3¾ oz.	O	16	22–21	113	97	3.0	334	476	1:1.4
	No. 60	¼ usual 22, 3¾ oz.	Sard	15	16–18	119	104	2.4	386	449	1:1.2
	No. 40	¼ usual 22, 3¾ oz.	O	15	25	107	92	2.6	332	485	1:1.5
	No. 25	¼ usual 22, 3¾ oz.	Sard	22	18	114	92	4.4	470	565	1:1.2
	AVERAGE							3.1	380	494	1:1.3
Maine sardines	No. 43	No. ¼, 3¼ oz.	S	13	12	113	100	3.0	369	484	1:1.3
	No. 29	No. ¼, 3¼ oz.	O	9	10–12	113	104	4.0	396	550	1:1.4
	No. 41	No. ¼, 3¼ oz.	S	23	4–5	127	104	2.6	275	440	1:1.6
	No. 62	No. ¼, 3¼ oz.	S	18	6–4	130	112	2.7	354	498	1:1.4
	AVERAGE							3.1	348	493	1:1.4
California sardines in tomato sauce	No. 58	No. 1 oval, 15 oz.	—	97	6	444	350	2.9	334	410	1:1.2
	No. 20	No. 1 oval, 15 oz.	—	87	6	420	333	2.2	344	408	1:1.2
	No. 15	No. 1 oval, 15 oz.	—	80	6	440	360	2.2	340	435	1:1.3
	AVERAGE							2.4	339	417	1:1.2
Japanese sardines in tomato sauce	No. 31	No. 1 oval, 15 oz.	S	97	7–8	466	369	2.4	423	460	1:1.1
	No. 34	No. 1 oval, 15 oz.	—	71	11–12	463	392	2.9	390	448	1:1.2
	No. 35	No. 1 oval, 15 oz.	—	80	11	456	392	2.3	350	450	1:1.3
	AVERAGE							2.5	388	452	1:1.2
California boneless sardines	No. 16	½ oblong, 8 oz.	S	10 g. 420 H_2	—	253	201	2.0	137	229	1:1.7
Norway sardines in tomato sauce	No. 25	½ oval, 9 oz.	—	37	7–11	279	242	2.8	302	457	1:1.5
Sweden plain sardines	No. 11	¼ usual 22, 3¾ oz.	O	13	18	120	107	3.3	436	500	1:1.1

[1] The following abbreviations are used: O—olive oil; P—peanut oil; S—soybean oil; Sard—sardine oil.

amount of protein present. Apparently, age, sex, sexual maturity, and other factors affect the amount of phosphorus, for it has also been shown that a large loss of phosphorus occurs after spawning. A more complete discussion of the problems involved in determining the composition of fish will be found in the classical compilation of Vinogradov *(15)*.

The purpose of this investigation was to determine the extent of variation in the calcium and phosphorus contents of canned sardines prepared and processed in different parts of the world. In a study as limited as the one being reported, it would be impossible to consider all the variables affecting the calcium and phosphorus contents of canned sardines. Since sardines are a migratory fish, appearing in a given area only periodically in quantities suitable for canning and remaining in the area for only a short period of time during which the entire pack of sardines for the area is processed, it was decided that analyzing several brands from a given area would yield results typical of the pack from that area.

Canned sardines from Portugal,[e] Japan, Norway, Sweden, Maine, and California were analyzed. Although it is not possible to determine the species of fish after canning, it is possible to list the species most probably canned, using the distribution of the various species as a basis: Portugal—*Clupea pilchardus;* Japan—*Sardinops melanosticta;* Norway and Sweden —*C. harengus* or *C. sprattus;* Maine—*C. harengus;* California—*S. caerula (16)*.

EXPERIMENTAL

Sampling. Wherever possible three or more brands from a particular area were used. Four cans of each brand were selected at random and the contents drained free of oil or other packing media. Fish from these cans were combined, passed 3 times through a meat grinder or else ground in a blender for 3 minutes.

Approximately 10-g. portions were taken for analysis. Triplicate determinations were made on each brand.

Methods. The ash and ash solution for each determination were prepared according to the method described by the A.O.A.C. *(2)*. Calcium was determined titrimetrically according to the micro-method described by the A.O.A.C. *(2)*. Phosphorus was determined colorimetrically by the method described by Fiske and Subbarow *(7)*, and Lowry and Lopez *(11)* using a Coleman Universal Spectrophotometer, Model 14.

The ash, calcium, and phosphorus contents as well as information concerning can size, net weight, drained weight, oil type and weight are presented in Table 2. Table 3 presents similar data on several other canned fish products that were run incidentally to the study.

RESULTS AND DISCUSSION

As shown from the data, plain canned sardines (in oil), regardless of area of origin, are a good source of calcium and phosphorus, with a Ca/P ratio favorable for absorption of these elements. A similar statement could be made concerning sardines packed in tomato sauce. Actually, the same processes are used for these two products—only the packing medium is changed from oil to tomato sauce.

The importance of scales as it affects the calcium and phosphorus contents of canned sardines is illustrated when the data concerning the Portuguese plain sardines, Portuguese boneless sardines, and Portuguese skinless

[e] The Portuguese sardines used in this investigation were furnished by Instituto Portugues de Conservas de Peixe, Lisbon, Portugal. All other samples were purchased at local markets.

TABLE 3

Physical and chemical characteristics of some miscellaneous canned fishery products

Product	Sample code	Can size and net content	Oil		Av. No. fish	Av. total weight in grams	Av. drained weight	Ash %	Ca mg./100 g.	P mg./100 g.	Ca : P
			Type[1]	Av. quan. in grams							
Portuguese squid	No. 70	¼ oval 30—4½ oz.	O	39	....	118	79	3.5	38	174	1:5
	No. 137	¼ oval 30—4½ oz.	O	40	....	110	70	2.7	30	174	1:6
Portuguese fillets of anchovies	No. 57 (rolled)	⅛ oval—2 oz.	O	15	13	57	42	14.8	185	166	1:0.9
	No. 14 (flat)	⅛ flat—2 oz.	O	14	....	51	37	16.7	150	253	1:1.7
Portuguese tuna fish	No. LTI	¼ club 30—4½ oz.	O	17	....	127	110	3.5	21	230	1:11
California tuna fish	No. 8 (fancy)	No. ½—7 oz.	S	36	....	198	162	1.4	27	258	1:10
	No. 8a (chunk)	No. ¼ can—3¼ oz.	S	0	....	88	88	1.3	19	224	1:12

[1] Abbreviations used are: O—olive oil; S—soybean oil.

and boneless sardines are compared. The average calcium contents are, respectively, 580, 490, and 54 mg./100 g. The average phosphorus contents are, respectively, 624, 545, and 320 mg./100 g. The average ash contents are 3.7, 3.0, and 1.9, respectively. These results show that the majority of the calcium contents and approximately 50% of the phosphorus and ash contents are present in the skin and scales. Approximately 16% of the calcium content and approximately 12% of the phosphorus content present in the plain sardines are lost when the bones are removed and the product is packaged as boneless sardines. An additional 75% of the calcium originally present and an additional 36% of the phosphorus originally present is lost when the skin and scales are removed.

Norwegian and Maine sardines have ash, calcium, and phosphorus contents almost identical, probably indicating the use of the same species, but lower than that of the Portuguese sardines. However, Maine sardines were much larger fish, averaging approximately 15 g. per fish whereas Norwegian sardines averaged about 5 g. per fish. It is possible that compensatory factors such as ecological conditions and sexual maturity might account for the similarity in mineral content and overcome differences caused by scaling. It should be noted that the larger fish in the Maine packs had much lower ash contents than the smaller fish indicating the effects of scaling of the fish prior to packing. Two brands of Norwegian sardines also had lowered ash contents. Whether this is indicative of scaling of the fish is not known, although it is not the general practice in the caning of such small fish as were present in the Norwegian packs. However, it is possible that the scales may have been removed accidentally during treatments prior to packaging.

California sardines in tomato sauce and Japanese sardines in tomato sauce had comparable calcium and phosphorus contents similar to those of the Maine and Norwegian plain sardines, but lower than the Portuguese sardines in tomato sauce. The ash contents of the California and Japanese packs were lower. The fish were large, and the presence of scales was not noted. The one brand of Norwegian sardines in tomato sauce examined, had fish of larger size with lowered ash content but with calcium and phosphorus contents comparable with the California and Japanese products.

The one brand of California boneless sardines examined had lowered ash, calcium and phosphorus contents when compared with the California sardines in tomato sauce. This reduction in the calcium and phosphorus contents of the California products was of a similar magnitude to the reduction noted in Portuguese boneless sardines as compared to Portuguese plain sardines or Portuguese sardines in tomato sauce.

SUMMARY

Calcium and phosphorus contents of 42 different brands of canned sardines were determined. These products originated in Portugal, Japan, Norway, Sweden, Maine, and California. Plain sardines, boneless, and boneless and skinless sardines and sardines packed in tomato sauce were included. The data showed that differences do exist in similar types of packs depending upon place of origin, most probably as a result of differences in species utilized.

Differences of much greater magnitude were found to exist between different styles of sardines from the same area of origin. Plain sardines had the highest calcium and phosphorus contents followed by the boneless sardines. Lowest calcium and phosphorus contents were found in the skinless and boneless sardines.

The importance of the skin and scales in affecting the nutritive value of canned sardines was discussed. Presence or absence of these formations alter the calcium and phosphorus contents to a greater extent than the presence or absence of the bone.

The ratio of Ca/P was shown to be approximately 1:1 in all of the brands examined regardless of country of origin except in the case of skinless and boneless sardines where the ratio approached 1:6.

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THE FATE OF INGESTED POLYOXYETHYLENE (20) SORBITAN MONOSTEARATE IN RATS

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The widespread use of synthetic emulsifiers in foods and pharmaceuticals makes it desirable to obtain as much data as possible regarding their metabolic fate. We have previously reported *(4, 5)* on the metabolism of sorbitan monostearate.[a] In this communication we report on the fate of polyoxyethylene (20) sorbitan monostearate after oral feeding to rats. The emulsifier was labeled with C^{14} in the polyol portion.

METHODS

Preparation of radioactive polyoxyethylene (20) sorbitan monostearate. Preparation consisted of condensing 2.3 g. (.00535 mols) of sorbitan monostearate (prepared from uniformly C^{14} labeled sorbitol (see *4)* with 4.7 g. (.107 mols) of ethylene oxide using 10 mg. of fused sodium oleate as catalyst. The reaction was carried out at 135-140°C. with shaking for 5 hours in a high pressure hydrogenation apparatus.

Table 1 compares the physical constants for the starting ester and the polyoxyethylene condensation product with constants obtained by us on a commercial sample.

TABLE 1

Comparison of physical constants of C^{14} labeled compounds with commercial preparations

	Sorbitan monostearate		Polyoxyethylene (20) sorbitan monostearate	
	C^{14} labeled	Span 60[1]	C^{14} labeled	Tween 60[1]
Hydroxyl No.	235	238	116	100
Acid No.	11	10	1	2
Saponification No.	156	151	46	48

[1] Kindly furnished by Atlas Powder Company, Wilmington, Delaware. Trade-mark name for sorbitan monostearate is "Span 60" and for polyoxyethylene (20) sorbitan monostearate is "Tween 60."

Animal treatment. Non-fasted male albino rats (250 g.) raised on Purina Laboratory chow were used. The emulsifier was given orally by stomach tube as a water solution. In the experiments in which the expired CO_2 was collected, the animals were placed in metabolism cages as previously described *(4)*. The expired CO_2 was collected for 48 hours in 3-hour intervals. In these experiments, however, C^{14} was found only in the samples collected in the first 12 hours. The urine and feces were collected separately as single samples at the termination of the experiment. The intestinal tract contents were included with the feces. C^{14} content was determined by radioactivity measurements, but no attempt was made to chemically identify the C^{14} bearing substances.

Thoracic duct fistula.[b] The surgical procedure used in the cannulation of the rat thoracic duct lymph is that described by Bollman *et al. (1)* except that the animals

[a] Trade-mark name for sorbitan monostearate is "Span 60," and for polyoxyethylene (20) sorbitan monostearate is "Tween 60."

[b] We are indebted to Miss Rita L. Paldino of the Department of Physiology, University of Southern California, for these preparations.

were stomach-tubed with vegetable oil (1.0 cc) 1½ to 2 hours before the operation. The animals were then placed in restraining cages for the duration of the experiment. The radioactive emulsifier was administered about 6 hours after the operation. The thoracic duct lymph collected in these experiments includes the intestinal and liver lymph.

Measurement of C^{14} radioactivity. All radioactive determinations were carried out at infinite thickness on $BaCO_3$ obtained directly or by wet combustion, or by direct counting, using a gas flow counter. In order to reduce the counting error to a minimum (± 2%), 3 planchets were made from each fraction and counted to at least 5000 counts. All results were then averaged.

RESULTS AND DISCUSSION

The distribution of C^{14}, determined by radioactivity measurements, 24 and 48 hours after administration of polyoxyethylene (20) sorbitan monostearate (polyol labelled) is shown in Table 2.

TABLE 2

Data showing the % recovery of C^{14} after oral administration to rats of C^{14} labeled polyoxyethylene (20) sorbitan monostearate (labeled in polyol portion). Comparable experimental data previously obtained with sorbitan monostearate included for purposes of comparison

	Polyoxyethylene (20) sorbitan monostearate					Sorbitan monostearate[1] (polyol labeled)	
Exp. No.[2]	1	2	3	4	5	6	7
Emulsifier administered—g./kilo	0.25	0.5	1.0	1.0	1.0	1.2	6.5
Urine	9	7	6	7	10	25	16
Stool	67	93	73	77	87	48	66
CO_2	7	2	3	....	....	15	14
Lymph fluid[3]	....	....	....	0.2	0.3	....	....
Tissues[4]	....	....	....	....	....	....	3
Total recovery	83	102	82	84.2	97.3	88	99

[1] Data taken from previous publication *(4)*.
[2] The animals in Experiments 1, 2, 3, 6, and 7 sacrificed 48 hours after the feeding, and in experiments 4 and 5, 24 hours after the administration of the emulsifier.
[3] Volume of lymph fluid collected over the 24-hour period in Experiments 4 and 5 were 44 and 18 ml., respectively.
[4] Determination of C^{14} in tissues not run in Experiments 1-5 inclusive since total absorptions were so low as to preclude sufficient radioactivity in tissues to allow measurement.

It will be seen that the relative amounts of C^{14} in expired CO_2 and in urine is considerably less for the polyoxyethylene (20) monostearate than for the unmodified sorbitan monostearate, and the proportion of C^{14} in the stools is considerably greater than for sorbitan monostearate while the total recoveries are of the same order of magnitude. The modification of sorbitan monostearate by reaction with ethylene oxide thus produces a great change in the metabolic pattern.

The high total recovery of C^{14} (82 to 102%) which is considered excellent in this type of experiment indicates practically complete elimination of the polyol moiety from the animal after oral feeding. These results are in line with those obtained in human feeding studies of a related emulsifier, polyoxyethylene (20) sorbitan monooleate *(2)*. In these studies 4-6% of the polyol moiety was accounted for in the urine, with 90-98% recovered in the feces.

Although the present studies show that most of the polyol moiety of polyoxyethylene (20) sorbitan monostearate is eliminated after oral feed-

ing, it was felt important to determine if any of the C^{14} bearing substance was absorbed from the intestinal tract into the lymphatic system (as fat is absorbed in the intestines) and re-excreted before elimination. The data in Table 2 also show (Exp. 4, 5) that a negligible amount of the fed C^{14} is recovered in the lymph fluid over a 24-hour period after ingestion of the emulsifier. It is therefore concluded that the process of absorption and re-excretion, if it does occur, does not go by the lymphatic route.

It has been previously shown *(3)* and *(6)* that the hexitols—sorbitol and mannitol—are readily oxidized by intact animals after oral administration, and in previous experiments with polyol labelled sorbitan monostearate *(4)* (Exp. 6 and 7, Table 2) fed as a water emulsion, that 14 and 15% of the C^{14} was recovered in the expired air.

In analyzing the data in Table 2 and attempting to account for the marked difference in metabolic pattern between sorbitan monostearate and polyoxyethylene (20) sorbitan monostearate, the chemistry and composition of these emulsifiers should be kept in mind. Sorbitan monostearate is not a homogeneous compound but consists of several partial fatty esters of sorbitol and sorbitol anhydrides, and some free polyol. Its complex composition is shown by the chromatography *(4)* of the polyols obtained by saponification of the commercial emulsifier[c] which has shown the polyol to be a mixture of 14% hexitols, 70% hexitans, and 16% hexides.

Polyoxyethylene (20) sorbitan monostearate is produced by reacting 20 mols of ethylene oxide per mol of sorbitan monostearate. This results in polyoxyethylene chains attached by ether linkages to the polyol portion and leaves no unchanged sorbitol or sorbitol anhydrides. The average molecular weight of the polyol portion is increased about 6-fold by the addition of the ethylene oxide.

It is quite likely that this increase in molecular weight would result in decreased absorption in the intestine, and thus accounts for the lesser amounts of C^{14} in urine and expired CO_2, with concomitant increase in amount of C^{14} in the feces.

SUMMARY

A method has been described for preparing in small quantities polyol labelled (C^{14}) polyoxyethylene (20) sorbitan monostearate.

The oral administration of the above substance to rats showed that 6 to 10% of the polyol moiety is excreted in the urine and 2 to 7% recovered in the expired CO_2. The remainder of the polyol moiety was recovered in the feces. Negligible amounts of the C^{14} were recovered in the lymph fluid indicating that the amount of polyol moiety recovered in the feces had passed through the intestinal tract without absorption into the vascular system.

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[c] Span 60.

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THE EFFECT OF SUNLIGHT ON CRUDE LIPIDS EXTRACTED FROM FRESH AND FROZEN VEGETABLES[a, b, c]

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It is known that light causes the development of peroxides in oils *(4)*, and in unsaturated fatty acids which are mixed with chlorophyll previous to exposure *(3)*. Autoxidation of certain oils in the presence of chlorophyll has been reported *(2, 11)*. Past work at the Geneva Station gave indication that vegetable crude lipids were sensitive to sunlight. Dr. A. C. Wagenknecht of the Geneva Laboratory obtained some evidence of this also, in work with extracts from dehydrated peas. It was shown in these laboratories *(6)* that carotene reduces drastically the formation of peroxides by chlorophyll in vegetable oils when exposed to direct sunlight. Because of these facts, a study was undertaken to determine the effect of sunlight on the crude lipids extracted from vegetables.

MATERIALS AND METHODS

Six vegetables were employed: asparagus, Martha Washington variety; sweet corn, Golden Cross variety; lima beans, Fordhooks; peas, Thomas Laxton variety; peas, purchased on the market, probably a Telephone type; spinach, Nobel and Heavy Pack varieties; and snap beans, Tendergreen variety. Blanched and unblanched samples were held in frozen storage at 0°F. (–17.8°C.) for varying periods of time. In addition to these, freshly harvested and unblanched and the corresponding blanched samples of lima beans, peas, and sweet corn, of the same varieties noted before, were frozen overnight. This was followed by immediate lyophilization and extraction to determine differences which might be found when the lipids from fresh and stored materials were exposed to the action of sunlight.

The vegetables were dried by lyophilization and the crude lipids prepared as described in previous publications *(5, 7)*.

Twenty-milligram (± 10%) samples were distributed as evenly as possible in white porcelain evaporating dishes, 100 mm. in diameter, from ethyl ether solutions of the crude lipids. The ether was evaporated by swirling. These operations were conducted in the dark or in dim red light.

The samples were exposed to bright mid-day sunlight (10,000 foot candles in intensity), as measured by a Weston illumination meter model 603, reading from 0 to 10,000 foot candles, or an instrument calibrated to be its equivalent, for varying periods of time from 0 to 120 minutes exposure. Cloudless days were selected for this work. Ambient temperatures were taken during most of the exposures. Some samples were exposed under glass to cut out the shorter ultraviolet rays.

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[b] Approved by the Director of the New Jersey Agricultural Experiment Station for publication.

[c] Approved by the Director of the NYS Agricultural Experiment Station for publication as Journal Paper No. 1018.

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Following the exposures, the samples were analyzed for peroxides, and calculated as peroxide numbers, as described previously *(5, 7)*.

Determinations of total chlorophyll and carotenes were made in the crude lipid extracted from both raw and blanched peas, lima beans, and spinach by modification of methods given in the literature *(1, 9, 10)*. The crude lipids were dissolved in petroleum ether. The solutions resulting from peas and lima beans were passed through a column of magnesium oxide and Super-Cel (1:3) to separate the chlorophyll from the carotenes. The solutions of spinach crude lipid were separated on columns of magnesium oxide and Super-Cel (1:9). The chlorophyll samples were prepared with 90% acetone for reading in the Beckman DU spectrophotometer while the carotenes were made to volume with petroleum ether containing 3% of acetone. In addition to this, absorption spectra of the crude lipids of several of the vegetables used in this study were made, from 2400 Å to 7000 Å. These should be considered qualitative rather than quantitative.

A Beckman recording spectrophotometer was used to obtain the curves in Figures 3, 4, and 6. Ethyl ether was used as the solvent for the lipids in the absorption spectra work.

RESULTS AND DISCUSSION

The results of this investigation are presented in Tables 1 and 2, and in Figures 1 through 6.

An examination of Table 1 discloses significant differences in the formation of peroxides in the unblanched samples and those of the corresponding

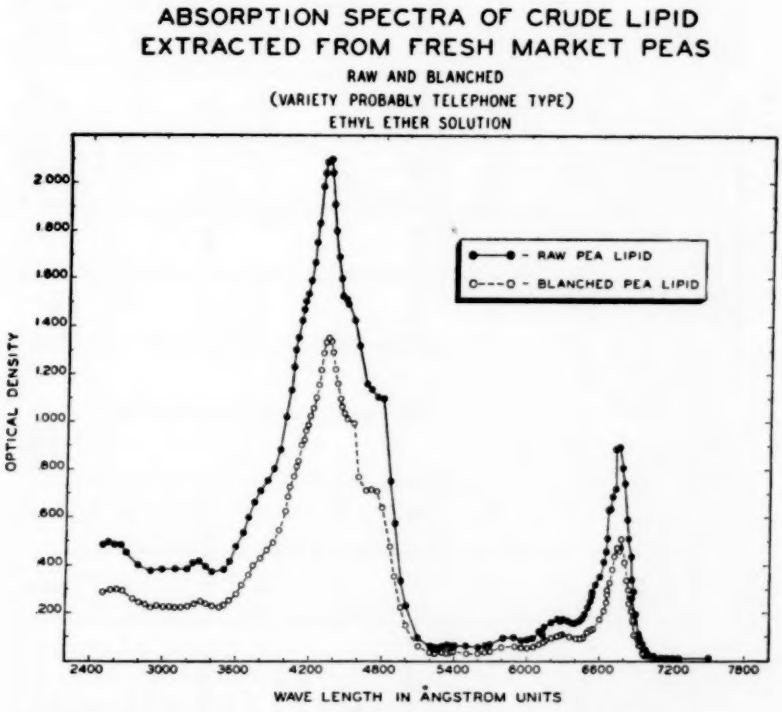

Figure 1

TABLE 1

Sunlight studies—development of peroxides in vegetable lipids

(All exposures in white porcelain evaporating dishes; 10,000 foot candles, sunlight intensity)

Source of lipid and ambient temperature	Storage at 0°F. years	0 min.	1 min.	2 min.	5 min.	10 min.	15 min.	30 min.	45 min.	1 hr.	1½ hrs.	2 hrs.	Fifteen minutes exposure and 1½ hours in dark before titrating except as otherwise indicated		Two hrs. under glass except as otherwise indicated
		Peroxide numbers[1]													
4286															
Asparagus, raw	4	6	98	457	1434	1719	1450	1647	1573	1271	1170	862	1 minute exposure	129***	
Asparagus, blanched	4	0	21	.....	97	476	1292	1220	1055	934	761	612	5 minutes exposure	98***	
4072															
Beans, lima raw, 23-28°C.	6	5			914	1672	1788	1830	1923	1920	1696	1687	5 minutes exposure, 2½ hours in dark	1008	
4072															
Beans, lima 33-40°C., blanched	6	0			716	1812	1965	1707	1762	1628	1510	1289	2¾ hours in dark	792	
Beans, lima raw-fresh 38-42°C.	0	0			.....	.....	1532	1714	1662	1536	1455	1401	3 hours in dark	1275	1630****
Beans, lima, 1953 fresh, blanched 21-24°C.	0	0			436		1758	2142	1827	1801	1599	1681	5 minutes exposure	534***	1734****
4332															
Beans, snap, raw	1	Trace			434	994	1310	1207	1307	1280	979	809		538***	1205****
4332															
Beans, snap blanched	1	0			20		89	260	700	761		931			
4341															
Beans, snap					4 min.						77 min.				
raw-fresh	0	0	Trace		16	35	62	294	451		607	643			
blanched	0	0	Trace		3	.....	19	73	89	209	466	635			

TABLE 1 (concluded)

4313 Corn, raw 42-44°C.	3	26					108	220	234	379	479	603		
4313 Corn, blanched 28-33°C.	3	12					7	23	91	143	260	369	10***	325*****
4336 Corn, fresh raw, 38-45°C.	0	0					15	24	44	148	474	569		
4336 Corn, fresh blanched 26-29°C.	0	0					4	7	8	11	17	31	30 minutes exposure, 2¼ hours in dark	6	13*****
4320 Peas, raw 30-40°C.	2	46					789	1598	1645	1767	1372	1307		1616***	
blanched, 11-14°C.	2	0			80	126	336	1427	1674	1804	1566	1639	5 minutes exposure	65***	
4340 Peas, fresh, raw	0	0	18	39	228	1521	.....	1560	1845	1460	1063	755			
blanched, 18-27°C.	0	0	10	13	40	90	134	1085	1613	1500	1541	1679			
4327 Spinach, raw 23-26°C.	1	0			0		14	39	207	384	622*	634**	5 minutes exposure, 3½ hours in dark	Trace	
4327 Spinach, blanched 26-33°C.	1	0			0		0	0	0	0	16	21	5 minutes exposure, 2½ hours in dark	0	9*****

[1] All peroxide values are net values. If the blank had a peroxide number, it was subtracted from the exposed values before recording in this table.

* 8,000 ft. candles at end of 1½ hour period.
** 5,000 ft. candles at end of 2 hr. period.
*** 2 hours in dark before titrating.
**** 1 hour under glass.
***** 1½ hours under glass.

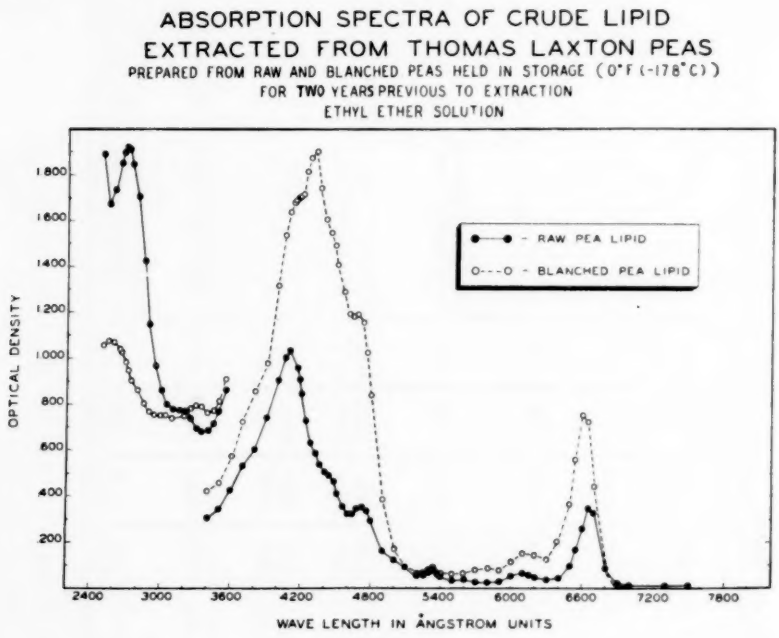

Figure 2

blanched samples. These differences are apparent in two ways. After varying periods of exposure to sunlight, the crude lipids from the raw vegetables gave a more rapid increase in peroxide number than those crude lipids which had been extracted from the corresponding blanched samples. Also, the peroxide numbers reached higher peaks for the unblanched material when contrasted with those obtained for the corresponding blanched material similarly exposed. In the case of the crude lipid extracted from lima beans, the reverse seems to be true. These observations hold for fresh as well as for the unblanched stored samples, even though the lipids of the latter had undergone changes in the unblanched samples during storage of the vegetable *(5, 7)*. The reason for the fact that the crude lipids extracted from blanched stored sweet corn yield substantially higher peroxide numbers on exposure to sunlight than the crude lipids extracted from blanched fresh sweet corn is obscure at the moment. However, it is known that peroxides, and, therefore, evidence of deterioration, were dcteeted in the crude lipid extracted from blanched sweet corn which had been stored for 1½ years *(5, 8)*. This was not the case in the crude lipids extracted from blanched samples of the other vegetables studied *(5, 7)*.

Samples exposed for relatively brief periods of time, followed by holding in the dark for 1 to 2¾ hours, gave values which indicated little, if any, increase in peroxides as the result of this treatment. There is little evidence that a delayed chain reaction of any sort is involved.

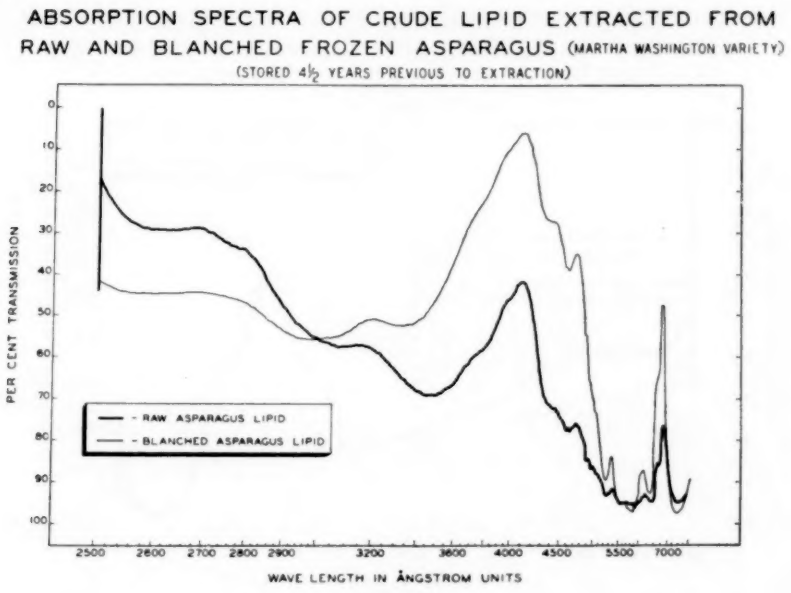

Figure 3

The problem of the wavelengths of light effective in producing peroxides in the lipid during exposure to sunlight was given consideration. At the time the samples of crude lipids were exposed to sunlight for varying periods of time, additional samples were exposed but were covered with Pyrex glass beakers. Substantially the same results were obtained for these samples as for the uncovered samples (Table 1). In view of the fact that glass cuts out most of the shorter ultra-violet waves the indication is that the main activity is not in that part of the spectrum. This suggests that the peroxide formation is brought about by the visible light rather than by that in the ultra-violet ranges. Final confirmation based on determination of activity of definite wave-lengths will be completed later.

The effect of temperature on the results seems to be relatively unimportant. However, a definite conclusion cannot be reached because the data are insufficient.

Data on the carotene and chlorophyll contents of the crude lipids from raw and blanched lima beans, peas, and spinach are shown in Table 2. The quantities of carotene present in these crude lipids is of a lower order of magnitude than that employed in the work with almond and Brazil nut oils *(6)* with the exception of that of blanched spinach. Since the spinach samples had been held in frozen storage at 0°F. (–17.8°C.) for 3¾ years, it is obvious that considerable loss of carotene occurred in the raw spinach *(13)*. It seems, therefore, that the low peroxide values found in the crude lipid from blanched spinach after exposure to sunlight can be explained by the activity of this rather large amount of carotene. It may be that the

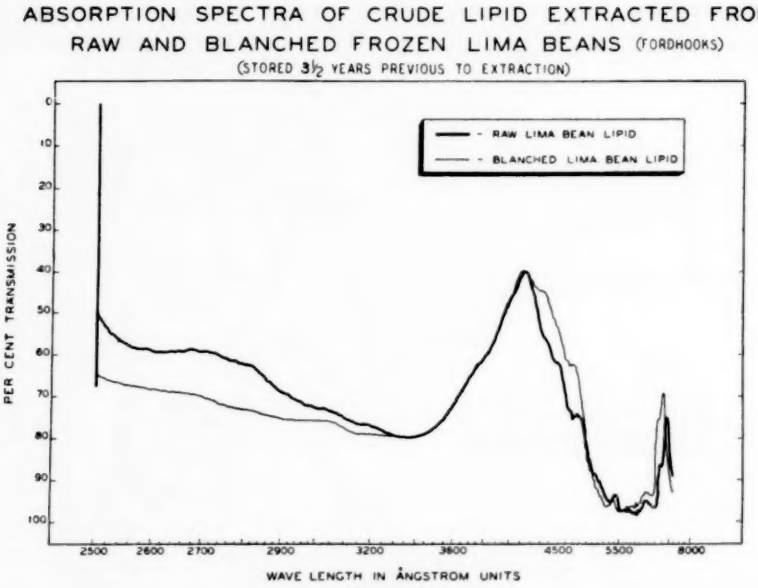

Figure 4

much smaller amounts of carotene present in the other samples were not great enough to affect the results so significantly. Also, the fact that the differences in the carotene contents of the raw and blanched samples of the other vegetables were not so great as in the spinach samples may be significant.

The absorption spectra information is shown in Figures 1 through 6. Characteristic peaks can be seen for chlorophyll and carotene in the blanched and fresh samples. The raw samples which had been held in frozen storage for periods of time usually had peaks to the left near 4200 Å, and to the right near 6600 Å of those obtained with the blanched and fresh samples. This is understandable because of the conversion of chlorophyll

TABLE 2
Carotene and chlorophyll content of crude lipids of peas, snap beans, and spinach

Vegetable	Storage 0°F.	Carotene content mcg./gm.	Chlorophyll content mg./gm.
Peas, raw	None	537.5	9.58
Peas, blanched variety—probably Telephone	Fresh	597.7	9.36
Lima beans, raw	3½ yrs.	384.8	1.73
Lima beans, blanched Fordhook		434.5	1.27
Spinach, raw	3¾ yrs.	682.4	22.56
Spinach, blanched Heavy pack, var.		8196.3	67.21

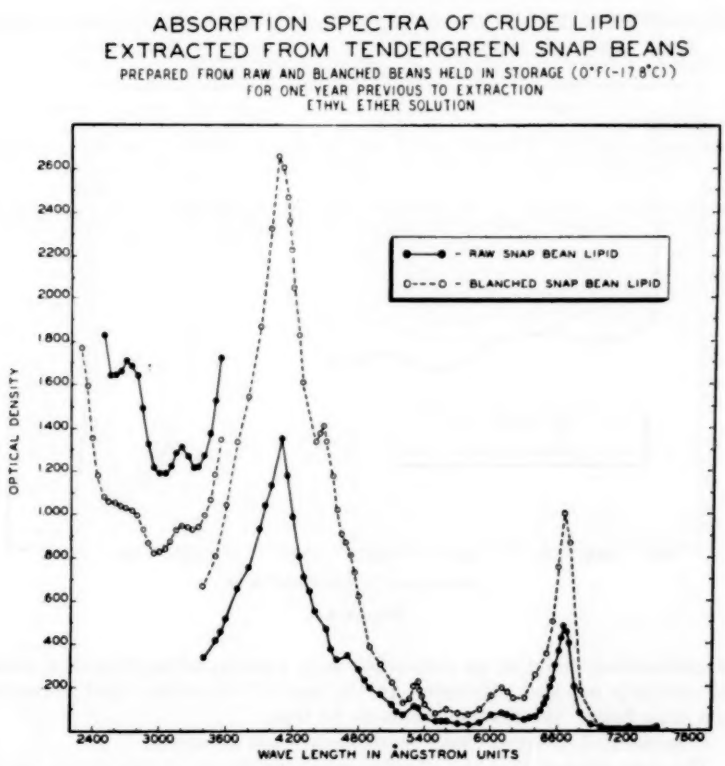

Figure 5

to pheophytin in the unblanched material during frozen storage *(12)*. These figures show also small pheophytin "bumps" at about 5300 Å for stored material both unblanched and blanched. The fact that they are not found in the fresh material, unblanched and blanched, suggests that pheophytin is formed during storage in the blanched as well as in the unblanched. Exposure to the sunlight caused a gradual disappearance of the pigments. Those lipids which were bright green in color changed to grayish-green in a few minutes, and partially disappeared after half an hour. After 1½ to 2 hours, the pigments had entirely or almost entirely disappeared.

SUMMARY

The effect of sunlight at 10,000 foot candles intensity on the extracted crude lipids prepared from certain vegetables was investigated. Frequently, after varying periods of exposure to sunlight, the crude lipids from raw vegetables gave a more rapid increase in peroxide number than those crude lipids which had been extracted from the corresponding blanched samples, except in lima beans. The peroxide values usually reached higher peaks

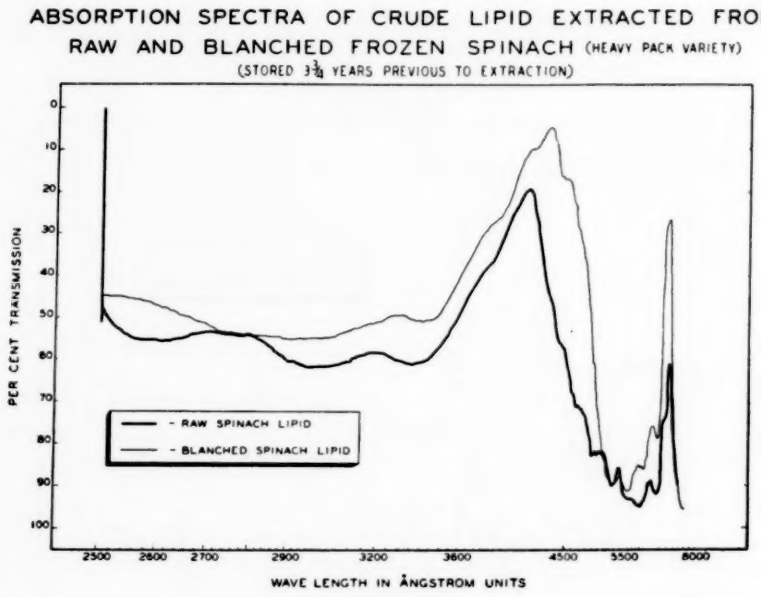

Figure 6

for unblanched material as contrasted with corresponding blanched material similarly exposed. However, in the case of the crude lipid extracted from lima beans, the reverse seems to be true.

It seems that delayed chain reactions are not involved.

The data suggest that the effective wavelengths are in the visible range.

The low values for spinach, especially the blanched material, can perhaps be accounted for by the presence of large amounts of carotene.

Absorption spectra of the samples were determined.

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EFFECT OF THE EGG SHELL MEMBRANE ON BACTERIA

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(Manuscript received October 17, 1955)

The shell membrane of eggs is an important protective barrier against microbial invasion. It is not clear, however, whether the membrane acts only as an inert mechanical barrier or whether it also actively destroys bacteria. The limited data of Stuart and McNally *(6)* suggested that the membrane is bactericidal since the addition of *Pseudomonas aeruginosa* cells to saline suspensions of membrane material resulted in destruction of 80% of the cells within 4 hours. This conclusion has been questioned, however, by Gillespie and Scott *(3)* because neither the effect of the saline alone on the bacteria nor the possibility that the reduction in numbers was due to adhesion of the bacteria to the membrane particles was considered.

In view of the importance of this problem for an understanding of the defensive mechanisms of the shell egg, the effect of the membrane on bacteria was re-examined.

MATERIALS AND METHODS

Preparation of membrane suspensions. Four large AA hen eggs were immersed in 70% ethanol for several minutes and then flamed in order to destroy external microbial contamination. The shell membranes were removed, as completely as possible, and washed repeatedly with distilled water to remove any adhering albumen. The washed membranes were ground with 0.85% NaCl in a pyrex glass tissue grinder until a uniform suspension was obtained and the volume then was adjusted to 50 ml. with the saline solution. Aseptic technique was used in all of these operations. This is essentially the procedure of Stuart and McNally except that they used sand to grind the membranes.

Preparation of the bacteria. The quantitative experiments were made with *Pseudomonas aeruginosa* H3, *Pseudomonas fluorescens* 126, and *Salmonella oranienburg* 200E. Additional tests were made with *Salmonella typhimurium* TM-1, *Proteus vulgaris* P-11, *Streptococcus faecalis* 9790, *Aerobacter aerogenes* 2000, and *Escherichia coli* 451B. All cultures were grown in trypticase soy broth at 35°C. for 1 day and reached population levels of about 10^9 cells per ml. For the experiments, the broth cultures were diluted 1:10,000 or more in distilled water in order to obtain appropriate cell concentrations. At these dilutions, carry-over of nutrients with the inoculum was considered to be insignificant. Exact numbers of cells were determined by plating with trypticase soy agar.

Test methods. The saline suspension of membrane was distributed in 15-ml. amounts into test tubes and each tube was inoculated with 1 ml. of the diluted broth culture of the test organism. The inoculated tubes were incubated in a water bath maintained at 30°C. and were shaken at intervals to resuspend the sedimented membrane particles. The fate of the inoculated bacterial cells was followed by plating 0.1 or 1-ml. aliquots of the suspensions at intervals, generally, of 1 hour. Each treatment was at least in duplicate and in some instances in quadruplicate. The bacterial numbers in the graphs and table are averages. Variations in cell counts between replicates were usually small.

In another approach, 1 ml. of an undiluted bacterial broth culture was mixed with 15 ml. of trypticase soy agar. After solidification, one or two small strips of membrane, approximately ½ inch by 2 inches, were placed on the surface of the agar. The plates were incubated at 35°C. and examined periodically for possible inhibition of bacterial growth at the edges and in the vicinity of the strips of membrane.

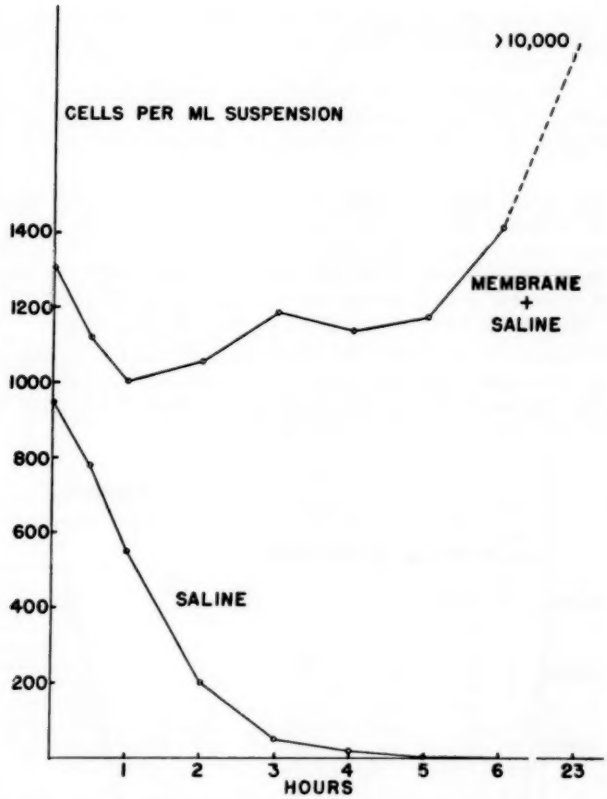

Figure 1. Survival of Pseudomonas aeruginosa in saline suspensions of shell membrane.

RESULTS AND DISCUSSION

As shown in Figure 1, *Ps. aeruginosa* decreased in number in the saline membrane suspension from 1,310 cells to a minimum of 1,010 cells per ml. during the first hour, a reduction of 23%. From 1 to 5 hours, the counts were intermediate between the above two values and after 5 hours the bacteria began to multiply and reached a level of more than 10,000 cells per ml. within 23 hours. These results differ from those of Stuart and McNally *(6)* only in that they obtained an 80% decrease in cell numbers compared to our 23%. The data, therefore, appear to indicate some destruction of the bacteria by the shell membrane. This interpretation, however, is not tenable in view of the results obtained with saline alone. In the latter, the death of the bacteria was much more rapid and extensive than in the membrane suspension. Eighty per cent of the cells were killed within only 2 hours and none could be recovered after 5 hours. The rate of bacterial destruction in the saline was probably even greater than indicated.

Although care was taken to inoculate essentially the same number of cells into both the membrane and control tubes, yet from the data in Figure 1 it would seem that the membrane suspension received 1,310 cells and the saline only 950 cells per ml. This difference, at least in part, is probably due to destruction of the bacterial cells in the saline during the interval of several minutes between inoculation and withdrawal of an aliquot for plating.

The data show, therefore, that the shell membrane is not bactericidal but on the contrary protects the bacteria against the toxic action of the suspending fluid; namely, the saline or physiological salt solution. This interpretation is in accord with the fact that eventually the membrane stimulates extensive growth of the bacteria by supplying them with nutrients, probably in the form of proteins and related compounds.

Results similar to those with *Ps. aeruginosa* were obtained also with *Ps. fluorescens*. The latter organism occurs more commonly on and in shell eggs. The data in Figure 2 show a reduction in cell count from 7,185 bacteria

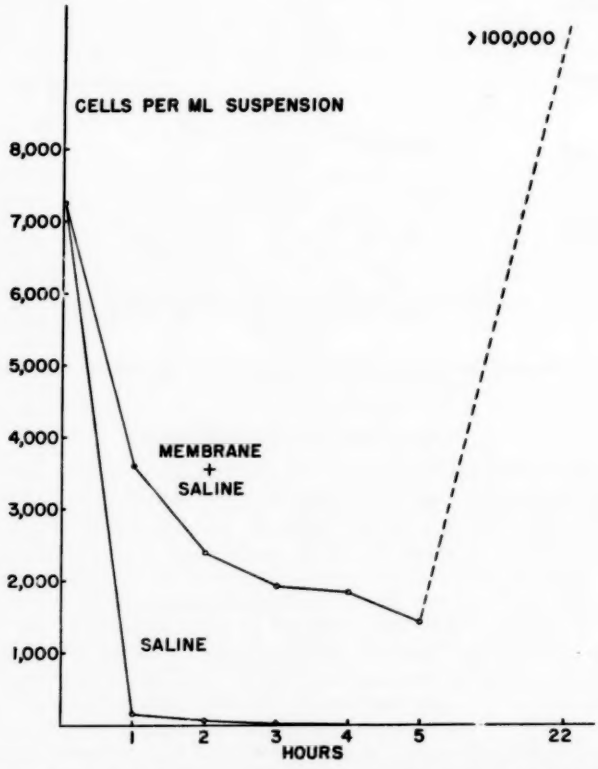

Figure 2. Survival of Pseudomonas fluorescens in saline suspensions of shell membrane.

to 1,433 bacteria per ml., or 80%, in the membrane suspension within 5 hours. These figures are similar to those obtained by Stuart and McNally *(6)*. However, destruction of the bacteria in the saline control was more rapid and reached 98% in 1 hour and 100% in 4 hours. Also, as with *Ps. aeruginosa*, the bacteria in the membrane suspension multiplied greatly after 5 hours and more than 100,000 cells per ml. were present after 22 hours of incubation. The shell membrane material, therefore, also protected *Ps. fluorescens* against destruction by saline and supplied nutrients for the growth of the bacteria.

The saline membrane suspensions ranged in pH from 8.7 to 8.9, whereas the saline itself was at pH 6.2 to 6.8. These differences in pH levels are not responsible for the trend in the results. For example, adjustment of the saline to pH 8.6 led to an even more rapid destruction of the bacteria than at pH 6.5.

The rapid death of the bacteria in saline is not due to the NaCl. Actually, as shown in Table 1, the bacteria die at a somewhat less rapid

TABLE 1

Rate of destruction of Pseudomonas fluorescens in various suspending media

Suspending medium	pH	Hours of incubation				
		0	1	2	3	4
		Cells per ml.				
Saline, distilled water	6.8	10,000	1,650	550	210	20
Saline, tap water	8.6	7,560	80	50	10	0
Distilled water	6.5	2,680	30	20	0	0
Tap water	8.7	3,740	20	30	0	0
Phosphate buffer, .05M	5.9	6,000	650	320	180	40
Phosphate buffer, .05M	7.1	2,450	1,480	60	0	0
Phosphate buffer, .05M	8.4	120	10	0	0	0

rate in saline than in distilled or tap water. Destruction occurs quickly also in .05*M* phosphate buffer at pH 5.9 and 7.1 and most rapidly at pH 8.4. Since all of the tubes in the experiment received the same amount of inoculum, the greater part of the large variations in counts at zero time is due to the death of the bacteria in the interval between inoculation and plating.

The inability of many types of bacteria to survive for any appreciable time in water or physiological salt solution has been known for many years. Palgen *(5)* has shown that *Ps. aeruginosa* dies quickly in physiological salt solution, so that only 50% of the inoculated cells are viable after 4 hours and only 20% after 8 hours. Similar results were also obtained with *Bacillus mesentericus*. Likewise, Winslow and Brooke *(7)* reported that washed cells of *Bacillus cereus, Bacillus megatherium* and *Serratia marcescens* decrease to less than 1% of the original population within one hour when placed in distilled water or physiological salt solution. Bacterial destruction could be prevented, however, by the addition of small amounts of peptone or meat extract to the suspending fluid. In our experiments a similar protective action was exhibited by the shell membrane. Butterfield *(1)* also found that the number of bacteria in natural waters may decrease

as much as 60% in 30 minutes when diluted with distilled water. More recently, Gunter *(4)* has shown that *Pseudomonas fluorescens* and other bacteria die rapidly in water and phosphate buffer solutions.

Not all bacteria, however, die quickly when placed in water. For example, *Escherichia coli* and *Salmonella typhosa* will remain viable in tap or distilled water for many hours without decrease in numbers *(2, 4)*. This fact suggested to us the possibility that with organisms resistant to water and saline there would be no significant decrease in viable cells when placed in saline or saline membrane suspensions provided the membrane itself is not bactericidal. This is exactly what was found, experimentally, with *Salmonella oranienburg*. It can be seen in Figure 3 that over a 5-hour period there was no appreciable change in cell numbers in either the membrane suspension or the saline control. After this period the bacteria began to multiply in the membrane suspension and to die in the saline so that after 28 hours, the former contained more than 100,000 bacteria per ml. whereas no viable bacteria could be recovered from the saline.

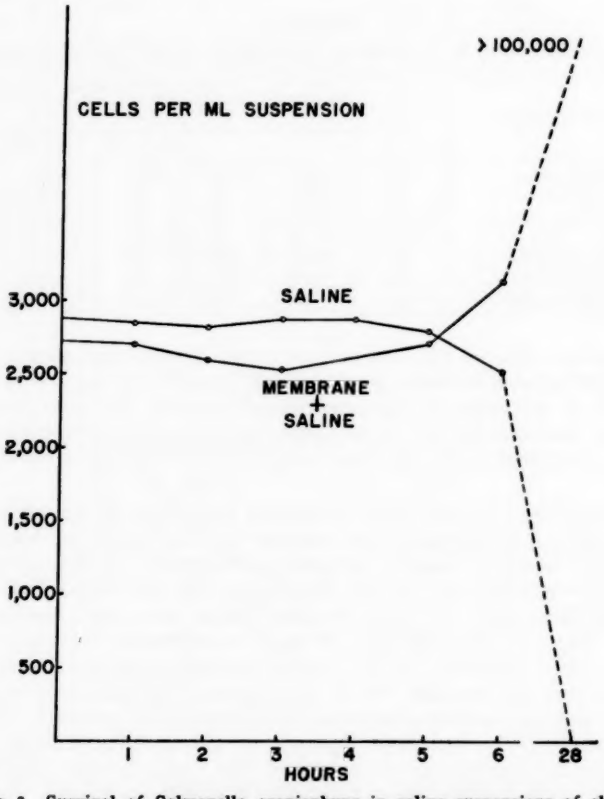

Figure 3. Survival of Salmonella oranienburg in saline suspensions of shell membrane.

The experiments in which strips of membrane were placed on agar plates seeded with cultures of *Ps. fluorescens*, *S. oranienburg*, *S. typhimurium*, *P. vulgaris*, *S. faecalis*, *A. aerogenes*, and *E. coli* also failed to give any evidence of inhibition of bacteria in the vicinity of the strips.

SUMMARY

There is no evidence from our experiments with *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella oranienburg* and other bacteria that egg-shell membrane is bactericidal. The apparent bactericidal activity of saline suspensions of membrane is due to destruction of the bacteria by the physiological salt solution in which the membrane is suspended. Actually, the membrane protects the bacteria somewhat against the toxicity of saline or, more specifically, water, and also supplies the bacteria with sufficient nutrients to permit extensive growth.

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Notes and Letters

A STUDY OF THE PROTEINASES OF LIPASE B[a]

Using the gelatin viscosity method of Northrop and Hussey *(3)* as modified by Koch and Ferrari *(2)*, it was possible to differentiate between two different proteinases in a fungal enzyme concentrate called lipase B. This investigation was made because of the peculiar shape of the pH activity curve obtained in the original investigation of different fungal proteinases *(2)*. In this paper it was stated that the results with lipase B indicated that two enzymes were active on the gelatin substrate.

The data given in the present note clearly show the existence of two distinctly different proteinases. They differ not only in their pH optima, but also in their stability to heat and cold.

Procedures. The reaction mixture for measuring proteolytic activity consisted of 10 ml. of a 5% bacteriological gelatin solution and 1 ml. of a solution of the fungal concentrate (containing 12.5 mg.). Blank viscosities were measured by substituting 1 ml. distilled water for the enzyme solution. Five ml. of the reaction mixture were pipetted into an Oswald viscosimeter immersed in a constant temperature water bath held at 40° ± 0.01°C. for measuring changes in relative viscosity.

Proteinase activity of lipase B was measured at various pH levels using 0.185 M buffered gelatin solutions. The buffers of Clark and Lubs *(1)* were used for neutral and alkaline pH levels and sodium acetate–acetic acid buffer solutions for the acidic range.

Observations. The pH activity curve of a freshly prepared solution of lipase B is presented in Figure 1 and shows two peaks indicating the presence of two different proteinases, one with an optimum at pH 4.7, called proteinase "f," another with an optimum at pH 7.5, called proteinase "s."

At their pH optima, the proteinases of freshly prepared solutions of lipase B (Figure 1) showed markedly different stability to heat and to storage at 0°C. Heating in a 0.2 M phosphate buffer at pH 7.1 for 30 minutes at 60°C. completely inactivated both proteinases. Heating in a 0.2 M acetate buffer at pH 4.7 for 30 minutes at 60°C. completely inactivated proteinase "s," but proteinase "f" was quite stable under these conditions. It is interesting that the pH optimum of proteinase "f" shifted from pH 4.7 to pH 4.2 under these conditions, accompanied by an apparent increase in activity.

A solution of lipase B buffered at pH 4.7 and held near 0°C. for 22 days also showed a shift in pH optimum for proteinase "f." It had even greater activity at pH 4.2 than the solutions heated at 4.7 (Figure 1). Thus, heating may have caused some inactivation of proteinase "f," but in terms of relative viscosity, the amount of inactivation was less than 6%.

When a solution of lipase B was held at pH 4.7 near 0°C. for 22 days, proteinase "s" showed a loss in activity compared with the fresh water solutions of lipase B (Figure 1). The loss was not nearly so great as that

[a] Prepared by Rohm and Haas Company, Philadelphia, Pennsylvania.

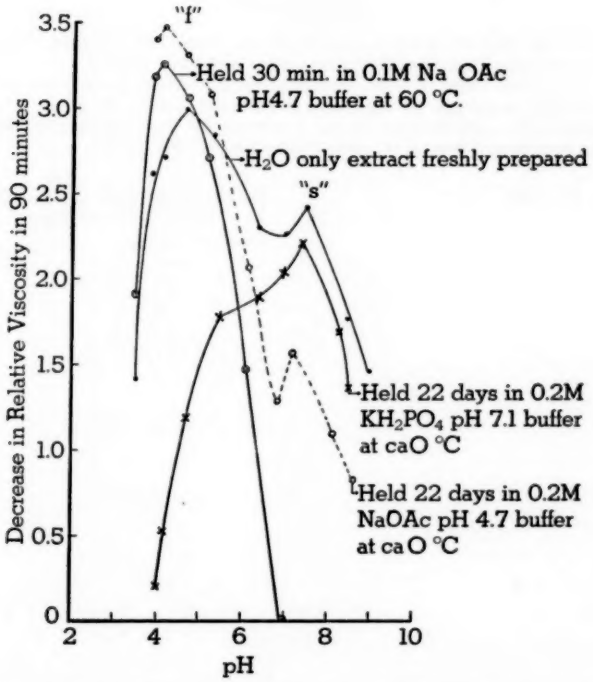

Figure 1. pH activity curves for the proteinases of lipase B. (Reaction mixture 4.55% gelatin and 12.5 mg. enzyme concentrate per 11 ml., temperature 40°C.)

shown for proteinase "f" when a lipase B solution was stored at pH 7.1 near 0°C. for 22 days. Under the storage conditions just noted, proteinase "s" retained 68% and proteinase "f" retained less than 10% of the activity of these proteinases stored at their respective pH optima. Thus, proteinase "f" possesses marked stability toward moderate heat at its pH optimum, while proteinase "s" is readily inactivated by similar treatment at its pH optimum. Proteinase "f," on the other hand, is less stable when stored near 0°C. at pH 7.1 which is the optimum of proteinase "s." However, proteinase "s" is relatively stable when stored at 0°C. near the pH optimum of proteinase "f." It is also apparent from Figure 1 that, at their respective pH optima, proteinase "f" is considerably more active than proteinase "s."

Neither of the proteinases were affected by heavy metal reagents such as KCN or NaF, nor by oxidizing or reducing agents. In this respect they are similar to other fungal proteinases, but they differ from *Aspergillus oryzae* type fungal concentrates in that the lipase B proteinases have quite a sharp pH optima as compared to the broad optimum near neutrality shown by *Aspergillus oryzae* type *(2)*.

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November 30, 1955

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Chicago, Illinois

DIFCO

Bacto-Dehydrated Media for Microbiological Assay of

VITAMINS and AMINO ACIDS

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BACTO-RIBOFLAVIN ASSAY MEDIUM
BACTO-NIACIN ASSAY MEDIUM
BACTO-THIAMIN ASSAY MEDIUM
BACTO-PANTOTHENATE ASSAY MEDIUM
BACTO-B_{12} ASSAY MEDIUM USP
BACTO-CS VITAMIN B_{12} AGAR
BACTO-FOLIC ACID ASSAY MEDIUM
BACTO-PYRIDOXINE ASSAY MEDIUM
BACTO-BIOTIN ASSAY MEDIUM
BACTO-CHOLINE ASSAY MEDIUM
BACTO-CF ASSAY MEDIUM
BACTO-TRYPTOPHANE ASSAY MEDIUM
BACTO-LEUCINE ASSAY MEDIUM
BACTO-METHIONINE ASSAY MEDIUM
BACTO-LYSINE ASSAY MEDIUM
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BACTO-ARGININE ASSAY MEDIUM
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The method employed in carrying stock cultures of the test organisms and preparing the inoculum for microbiological assay is important. The following media have been developed especially for carrying stock cultures and for preparation of the inoculum:

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